Reproductive isolation between anadromous and freshwater threespined sticklebacks (*Gasterosteus aculeatus*): insights from a hybrid zone.

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Thesis presented for the degree of
Doctor of Philosophy
at the University of Edinburgh

2005

Sticklebacks in a jam jar. The threespine stickleback is particularly amenable to both field and genetic studies of evolution. These freshwater fish, from Site 6 on the River Tyne are being tagged, photographed, measured, and fin-clipped before being released back into the River.

ABSTRACT

How do new species form? Adaptation to divergent habitats is often accompanied by divergence in form and can lead to reproductive isolation and speciation. Studies of hybrid zones (where two divergent forms meet) can provide insight into the nature of reproductive isolation and hence factors affecting speciation. In estuaries throughout the northern hemisphere resident-freshwater forms of the threespined stickleback (*Gasterosteus aculeatus*) have undergone parallel divergence from their marine ancestors. Sticklebacks are useful for studies of reproductive isolation and speciation because divergent resident-freshwater and migratory anadromous forms exist in sympatry in the lower reaches of rivers. A great deal is already known about the evolutionary history of sticklebacks, however, little is known about reproductive isolation in wild populations. In this thesis, I set out to investigate the nature of reproductive isolation in an anadromous-freshwater stickleback hybrid zone located in the River Tyne, Scotland and to explore whether the same genes underlie variation in the same traits in different populations.

1. There was no evidence of morphotype-based assortative mating, in an experimental pond manipulation. Hybridisation between morphotypes occurred readily and this s due in part i to the tendency of freshwater females to mate with large males.
2. In contrast, there is some evidence of reproductive isolation existing in the River Tyne wild population, and might be due to either ecologically dependent assortative mating or early hybrid fry mortality. Premating isolation cannot be strong, however, since hybrid juveniles represented 33-39% of the sample from sympatric sites. There was no evidence of directional bias in hybridisation.
3. Evidence suggests that morphological and genetic differences between anadromous and freshwater sticklebacks in the River Tyne are being maintained by postmating isolation in the wild. Genetic hybrids had reduced probability of overwinter survival. There is also strong evidence for selection against intermediate morphotypes, and in the case of intermediate lateral plates, this selection was sex-biased, being stronger against females.
4. Statistical associations between loci and traits were detected and suggest both stability and flexibility in the underlying genetic control of morphological divergence in sticklebacks.

DECLARATION

The work presented in this thesis is my own, apart from where otherwise acknowledged in the text, and the thesis has been written by myself.

Felicity C. Jones

ACKNOWLEDGEMENTS

This thesis would not have been possible without the assistance of Culum Brown. Culum spent over 3 months of his time helping with field work, enduring Scottish winter weather, to help me catch and collect data on sticklebacks from the River Tyne. Without his encouragement, particularly on cold dark mornings, and his ability to work 18 hours a day, 7 days a week, the vast amount of data collected would not exist. For this I am truly grateful. I'd also like to thank Culum for his enthusiasm and support throughout various stages of experimental design, lab work, analysis, and his comments on manuscripts in various forms.

I am also grateful to both my supervisors, Victoria Braithwaite and Josephine Pemberton, for the support they provided throughout my PhD. Josephine provided fantastic laboratory facilities which enabled me to do massive genotyping stints. Victoria spent many hours organising a Home Office licence which enabled me to tag and release fish in the field. Both Victoria and Josephine provided valuable comments on various drafts of this thesis, and I am extremely appreciative of the time they have spent.

I'd also like to thank Loeske Kruuk for statistical advice, and Dan Nussey for his inexhaustible knowledge of SPLUS commands.

I owe a huge debt to my friends in ICAPB (IEB) who kept me going at various stages of my PhD, particularly while Culum was away. Kevin Connaghan, Alex Hayward, Carolyn Gardner, Fiona Burgess, Helen Simcox, and Phil Boulcot all provided assistance with either pond construction or field work or at various stages.

I am thankful to Jill Lovell and Andy Gillies, of the ICAPB Sequencing Service, who were extremely flexible and always happy to run hundreds of samples on the sequencing machine.

I am also grateful to the Royal Society who provided funding for genotyping, the Genetics Society who provided funding for fieldwork. I'd like to thank the following people who kindly gave permission to access the River Tyne: The East Lothian District Salmon Fisheries Board, The East Lothian Council, Scottish Natural Heritage, The River Tyne Trust, the owners/trustees of Tyninghame Estate, Knowes Farm, Phantassy Farm, The National Trust at East Linton, and Lennoxlove Estate.

Finally, I'd like to thank my sister, whose culinary packages from Australia provided me with extra motivation to finish.

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CHAPTER ONE

INTRODUCTION:

SPECIATION, SPECIES PAIRS AND STICKLEBACKS AS MODEL ORGANISMS

INTRODUCTION

Speciation is one of the most exciting and well-studied topics in the field of evolutionary biology. Studying the nature of speciation (e.g. tempo and mode) is important because it improves our understanding of biodiversity and influences conservation issues. If we understand the factors, processes and mechanisms affecting speciation we can utilise appropriate measures to preserve species and their evolutionary potential.

Since Darwin's pioneering thesis on the role of natural selection in speciation (1859), numerous studies have begun to reveal the underlying complexity of processes involved. However, much remains unknown about these processes, primarily due to the difficulty of studying something that occurs on a timescale longer than the typical research grant. Rather than observing speciation as it happens, this process must be inferred from studies of genetic and phenotypic divergence, and laboratory manipulations.

Further complexity stems from the difficulty of defining a species. Defining species is necessary for understanding evolution and forms an important part of documenting biodiversity. As the fundamental units of biodiversity, recognition of distinct species aids in impact assessment and planning. Species definitions are also fundamental to scientific research since the comparison of information requires exact knowledge of the species being studied. There are many different species concepts in the literature. From a taxonomic realism perspective, where species are regarded as true entities in nature and not simply pigeon-holes constructed by humanity, all species definitions are variations on the general concept of species as

evolutionary lineages ('generalised lineage concept' de Queiroz, 1997). The evolutionary species concept defines species as "a lineage evolving separately from others and with its own unitary evolutionary role and tendencies" (Simpson, 1951). A similar concept to this is the phylogenetic species concept, which uses monophyly as the defining species criterion (Eldredge and Cracraft 1980). Under this hypothesis, a species is a monophyletic group composed of the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent (Eldredge and Cracraft 1980). Phylogenetic species concepts such as those mentioned above appear easy to apply, but can be limiting because the distinctiveness of evolutionary lineages may collapse on secondary contact. Since genetic differences between two groups of organisms cannot be maintained unless interbreeding is prevented (Dobzhansky 1951), the requirement of reproductive isolation makes the biological species concept (BSC, Mayr 1942) popular with many evolutionary biologists.. Under the BSC, species are defined as groups of actually, or potentially, interbreeding natural populations that are reproductively isolated from other such groups. It should be noted that many other species concepts exist, primarily because no one concept is applicable to all cases. Lack of conformity to any one species concept is a common phenomenon in plants. For example, botanists are often faced with situations where largely divergent plant species are able to hybridise and produce fertile progeny (Arnold 1997). The application of species concepts therefore requires flexibility and should be performed by keeping the biology, as well as the purpose of classification, in mind (Mallet 2005). In this thesis, I am interested in factors maintaining or promoting divergence between two coexisting forms, and therefore apply the BSC which is based upon reproductive isolation.

MODES OF SPECIATION

The initial stages of speciation involve divergence in genetics, morphology, ecology or behaviour between two groups of individuals. There is strong evidence for population divergence occurring in allopatry (between spatially separate groups) and this mode of speciation is commonly invoked when a population's range has been split by a geographic barrier (Ridley 1997). There is also evidence for divergence occurring in sympatry (between spatially coexisting groups) although, in some cases sympatric speciation is difficult to distinguish from divergence in allopatry followed by hybridisation on secondary contact (Seehausen 2004). Population divergence might also occur in parapatry, where a continuous population is exposed to selection pressures along an environmental gradient. Since parapatric divergence results in a cline in the natural population it is difficult to distinguish this mode of speciation from allopatric divergence followed by secondary contact.

Divergence between allopatric groups is thought to be promoted by genetic drift, bottlenecks and founder effects (but empirical evidence for the latter two is rare, Barton and Charlesworth 1984). In addition, different environmentally-based selection pressures can also play a large role. In sympatric populations, the existence of resource polymorphisms (Skulason and Smith 1995), multiple environment niches, and the absence of interspecific competition may promote speciation. In these cases, divergence is thought to be promoted by intraspecific competition driven character displacement, and trade-offs in fitness (e.g. limnetic and benthic sticklebacks, *Gasterosteus aculeatus*, Bentzen and McPhail 1984, Schluter 1993, and pumpkinseed sunfish, *Lepomis gibbosus,* Robinson and Wilson 1996). Aside from natural selection, sexual selection may also play a role in

speciation and there is an increasing amount of evidence to support this (see Panhuis *et al.* 2001). In the absence of resource polymorphisms and species depauperate environments, sexual selection may even be strong enough to drive sympatric speciation itself (e.g. possibly driving divergence in damselfish *Acanthochromis polyacanthus* on the Great Barrier Reef, Australia, Kavanagh 2000). This is supported by theoretical models (e.g. Laland 1994, Takimoto *et al.* 2000) but it is still a topic of considerable debate (Panhuis *et al.* 2001). More commonly, it is likely that sexual selection acts in conjunction with natural selection to drive the divergence and the evolution of reproductive isolation (e.g. Wilson *et al.* 2000).

REPRODUCTIVE ISOLATION

Under the BSC, speciation is complete when two groups have evolved to be reproductively isolated. Reproductive isolation may be in the form of premating or postmating isolation. Premating isolation (or prezygotic isolation) exists when a 'barrier' prevents the fusion of gametes (e.g. assortative mate choice, or differences in breeding times or locations). Alternatively, two distinct forms of postmating isolation (or postzygotic isolation) can occur. Firstly, hybridisation between species occurs but the offspring may be inviable/infertile (e.g. due to genetic incompatibilities – reduced fitness due to *endogenous* factors). Secondly, hybridisation between species may occur, and the offspring may be viable, but hybrids may be of decreased fitness in alternative environments or niches (reduced fitness due to *exogenous* factors). The relative importance of premating and postmating isolation, and the factors underlying the type of reproductive isolation is a major area of focus in speciation studies and of this thesis.

FISH SPECIES PAIRS

Many fish species exist as species pairs (genetically, morphologically, and behaviourally distinct populations that are in sympatry for at least some part of their lifecycle (Taylor 1999)). Some fish species pairs are thought to have evolved in sympatry. For example, cichlid 'flocks' or radiations (*Tilapia* species) within small crater lakes in Cameroon are monophyletic (Schliewen *et al.* 2001) rather than paraphyletic, indicating a single origin. Bernatchez *et al.* (1996) used the term 'micro-allopatric speciation' to describe reproductive isolation which had evolved between whitefish (*Coregonus* species) as a result of the use of different foraging microhabitats. Microhabitat variation is also thought to be associated with cichlid radiations (*Pseudotrophius callainos)* in Lake Malawi (Rico and Turner 2002). In contrast, an allopatric or parapatric mode of speciation is more relevant to anadromous and freshwater fish species pairs evolving from marine ancestors (Taylor 1999). There is substantial evidence for the role of natural selection in promoting the formation of species pairs (see Schluter 1996 and Taylor 1999 reviews). Divergent selection is thought to have driven the formation of fish with different trophic morphs (e.g. limnetic and benthic arctic charr, *Salvelinus alpinus,* Malmquist 1992) and life history traits (e.g. anadromous and freshwater sockeye and kokanee salmon, *Oncorhynchus nerka,* Wood and Foote 1996). In addition, recent studies of reproductive isolation and mate choice have found evidence supporting the possible role of sexual selection in sticklebacks (*G. aculeatus,* Vamosi and Schluter 1999, Bakker *et al.* 1999, Boughman 2001), cichlids (*Amphilophus citrinellum,* Wilson *et al.* 2000), and salmonids (*O. nerka,* Craig and Foote 2001, *Salmo salar,* Landry *et al.* 2001). Lack of interspecific competition is also thought to be a key factor favouring the formation of species pairs (see

Robinson and Wilson 1994) and this may explain the association of many species pairs with recently deglaciated habitats (Bernatchez *et al.* 1996). Colonisation of these species-poor habitats by fish from marine and/or freshwater refugia may have resulted in resource or habitat specialisation within a species rather than between species.

A migratory life history (diadromy) may play an important role in the adaptive radiation of many freshwater fish. In the absence of strong natal homing, diadromy promotes dispersal, a broad geographic range, and inhibits genetic divergence and therefore speciation (McDowall 2001). Often diadromy is not obligatory and thus, in contrast to the homogenising effects of dispersal on genetic structure of the diadromous population, dispersal into new habitats by diadromous species which then fail to migrate (facultative diadromy) has a diversity-generating effect between resident freshwater populations. The adaptive radiation of many freshwater fish species is thought to be due to the diadromous life history of the ancestral form (e.g. New Zealand galaxiids, *Galaxias* species, McDowall 2001, sockeye salmon, *O. nerka,* Foote *et al.* 1994 and sticklebacks, *G. aculeatus,* McPhail 1994, Taylor and McPhail 1999, 2000). These anadromous (a form of diadromy involving migration from marine habitats into freshwater habitats to reproduce) and freshwater species pairs are thought to have evolved in the last 15,000 – 20,000 years. This relatively recent origin has brought some to suggest that, in sympatry, they are in the final stages of speciation (McKinnon and Rundle 2002, Taylor and McPhail 2000). For this reason, many fish species pairs provide an ideal opportunity to study adaptation, reproductive isolation, and speciation.

WHY STUDY HYBRID ZONES?

Hybrid zones, areas of contact where matings between genetically distinct groups of individuals occur, provide a useful setting for studies of speciation. The end result of hybridisation is often viewed as collapse of species distinction or, alternatively, reproductive isolation and speciation. However, many hybrid zones appear to be stable (e.g. fire-bellied toads, *Bombina bombina* and *B. variagata,* Szymura and Barton 1986, meadow grasshoppers, *Chorthippus parallelus,* Hewitt 1988). For the two groups to remain distinct entities, gene flow must be countered by selection against hybrid and/or recombinant individuals. In the absence of selection, hybridisation between two distinct species would eventually lead to collapse of the species distinction. By studying a hybrid zone, it is therefore possible to determine factors maintaining species divergence.

There is evidence to suggest that different factors may maintain divergence between two species in different locations. For example, endogenous selection (genetic incompatibilities) against hybrids may play a role in maintaining species differences in the hybrid zones of the fire-bellied toad located at Krakow and Przemysl (Kruuk *et al.* 1999). In contrast, in *Bombina* hybrid zones located at Pescenica and Apahida, strong associations between habitat and genotype have been observed suggesting that environmentally-mediated selection may play a more important role in maintaining species divergence (Vines *et al.* 2003). Exogenous selection based on environmental conditions outside the hybrid zone was also invoked to explain the maintenance of differences between anadromous and freshwater sticklebacks, since hybrids within the hybrid zone showed no sign of reduced fitness (Hagen 1967).

The position and shape of clines in traits and genes can also provide clues as to the type of selection maintaining divergence between species. Displacement in clines might be expected under a bounded hybrid-superiority model (Moore 1977), where hybrids are of superior fitness within the hybrid zone but show reduced fitness when dispersing outside the hybrid zone. In these cases, clines will closely track environmental gradients but may not coincide exactly. For example, displacement in clines in morphology, male sterility, and cuticular hydrocarbons has been observed in hybrid zones between subspecies of the meadow grasshopper (*C. parallelus*, see Buckley *et al.* 2003), although, it should be noted that patchy colonisation history might also explain the observed cline displacement. Inference about the strength of selection acting on a particular trait or allele can also be made from analysis of clines. In hybrid zones maintained by a balance between selection and dispersal, a neutral allele would be expected to introgress at a faster rate and thus have a wider cline than one under strong selection. Discordance in cline width may only be observed when hybrids are subject to weak selection due to weak linkage disequilibrium between unlinked markers. In the presence of strong selection acting on hybrids, linkage disequilibrium will have the effect of pulling clines in traits and alleles together (Szymura and Barton 1986). Hybrid zones also allow us to explore questions about the evolution of reproductive isolation, such as whether postmating isolation drives reproductive character displacement (e.g. strengthens mate discrimination) and leads to premating isolation (the theory of reinforcement, Dobzhansky 1951). Empirical support for reinforcement is equivocal (see Noor 1999 for a recent review). Females from a meadow grasshopper hybrid zone (where hybrid males are sterile) do not produce offspring of greater fitness when allowed to choose mates than when forced to mate randomly (Ritchie *et al.*

1992). In contrast, there is some evidence to support reinforcement as a possible mechanism driving premating isolation. A study of mate choice in limnetic and benthic sticklebacks (*G. aculeatus*) suggests that assortative mating is stronger in sympatric morphs than allopatric morphs (Rundle and Schluter, 1998). The continuum of trait distribution within a hybrid zone (unimodal to bimodal) may provide clues as to the importance of premating and postmating isolation (Jiggins and Mallet 2000).

In addition to investigating premating and postmating isolation and the selective forces acting against recombinant individuals, the high diversity of morphotypes and the presence of recombinant genotypes within a hybrid zone provide opportunities to explore the genetic architecture underlying morphological divergence between species, and thus the underlying genetics of adaptation (Rieseberg and Buerkle 2002). Identifying quantitative trait loci (QTL) and the genetics underlying trait variation is interesting not only because it enables us to understand how a particular mutation causes variation in a particular trait, but also because it allows us to investigate how genetic variation, and thus evolutionary potential, is maintained (Barton and Keightley 2002). It is becoming increasingly apparent that single mutations can explain substantial quantitative trait differences between species, and this is suggestive that species divergence can occur quickly and involve few mutations of large effect rather than gradual divergence involving many mutations of small effect. Evidence of rapid evolution on traits under heavy selective pressure are illustrated by transplantation studies in guppies (*Poecilia reticulata,* Reznick *et al.* 1997) and rapid loss of lateral plates in sticklebacks (Bell *et al.* 2004). In a recently colonised Alaskan lake population, the frequency of complete lateral plate

stickleback morphs declined by 89% over a period of 12 years (Bell *et al.* 2004). In a study of a hybrid zone between sunflower species (*Helianthus petiolarus* and *Helianthus annuus*), Reiseberg *et al.* (1999) were able to identify chromosomal rearrangements which contributed to reduced hybrid viability and therefore maintained isolation between the two species. Genetic incompatibilities also cause sterility in hybrids of the meadow grasshopper (Virdee and Hewitt, 1992) and sister species of *Drosophila* (see Wu *et al.* 1996). In many cases hybrid sterility is found only in the heterogametic sex, and suggests that epistasis between sex chromosomes may play a special role in the evolution of reproductive isolation (e.g. Haldane's Rule, Haldane 1922).

WHY STUDY STICKLEBACKS?

Of all the fish species pairs, threespined sticklebacks are particularly amenable to speciation studies because of their wide distribution, enormous diversity, and the ease of maintenance in laboratory conditions (Braithwaite and Odling-Smee, 1999). Furthermore, a vast amount is already known about the evolutionary history of stickleback species pairs and this has been aided in part by the enormous efforts put in to understanding the stickleback genome (e.g. Peichel *et al.* 2001, Colosimo *et al.* 2004, Cresko *et al.* 2004, Shapiro *et al.* 2004). Sticklebacks are euryhaline fish occupying remarkably diverse habitats ranging from open marine environments, to brackish waters, rivers, ponds, and drainage ditches throughout their holarctic distribution. Since the last glacial maximum in the Pleistocene (approximately 18, 000 years ago) sticklebacks have undergone an adaptive radiation associated with the colonisation of freshwater habitats. Genetic analyses suggest that freshwater sticklebacks have arisen independently and repeatedly (parallel

evolution) from marine populations (Withler and McPhail 1985, Taylor and McPhail 1999, 2000). Adaptation to these environments has resulted in an extraordinary diversity of forms. Considerable variation in body size, shape, ornamentation (plates, spines, girdles etc), sexual colouration, life history strategy (e.g. nesting environment), and behaviour has been observed across the northern hemisphere. Most of this diversity is seen between freshwater populations whilst marine sticklebacks have remained relatively unchanged (at least in shape and size) from their fossilised ancestors (Walker and Bell 2000). This is most easily explained by the relative stability and uniformity of the marine environment compared to the diversity and instability of freshwater ecosystems over geological time.

In several places throughout their distribution, stickleback species pairs can be found including lacustrine (benthic and limnetic morphs), estuarine (anadromous and freshwater morphs) and fluvial (river and lake morphs) pairs (McKinnon and Rundle 2001). All of these species pairs display divergence in morphology and in some cases life history strategies and behaviour. Although reproductive isolation has evolved in some threespine stickleback species pairs, at present taxonomically all threespine sticklebacks share the same latin binomial: *Gasterosteus aculeatus*. Studies of threespine stickleback species pairs have contributed a vast amount of knowledge to what processes may be influencing speciation. Benthic-limnetic and anadromous-freshwater stickleback species pairs have been particularly well studied and I discuss these further below.

BENTHIC – LIMNETIC STICKLEBACK PAIRS

The sympatric, lake dwelling 'benthic' and 'limnetic' stickleback morphs are the most well studied of the species pairs. The 'benthic' morph feeds largely on macrobenthos in the littoral zone while the 'limnetic' morph specialises in planktivory in deeper water (McPhail 1984). Morphologically, benthic morphs have a deeper, more robust body shape, fewer shorter gill rakers, and shorter jaw and snout length than limnetic morphs (McPhail 1984, 1992, 1993). Due to their feeding specialisations the morphs are segregated spatially within the lake except during the breeding season when limnetic morphs enter the littoral habitat to breed. There is some evidence to suggest that the limnetic and benthic morphs prefer different littoral zone microhabitats during the breeding season (Vamosi and Schluter 1999, Taylor and McPhail 1999). Despite occupying benthic and limnetic niches, not all stickleback species pairs are identical in extent or pattern of divergence (McPhail 1992). This suggests that local selection regimes or founding populations are different between lakes.

Evidence for distinct gene pools between benthic-limnetic species pairs exists. Significant difference in allozyme frequencies between the two morphs were found in two lakes (Enos and Paxton, McPhail 1984, 1992). These findings were later supported by analysis of mtDNA haplotype frequencies in Enos, Priest, Emily but not Paxton Lake (Taylor and McPhail 1999). Recent microsatellite analyses (Taylor and McPhail 2000) found significant differentiation between the morphs (F_{ST}) in all of the above four named lakes.

Assortative mating experiments conducted on Enos lake morphs established that benthic and limnetic fish prefer to mate with their own kind (Ridgway and McPhail 1984). This provides evidence that reproductive isolation may exist as a result of premating barriers. In the wild this choice may be facilitated by microhabitat preference, and evidence also suggests that morphology (size Kraak and Bakker 1998, and colouration, Milinski and Bakker 1990) and courtship behaviour (McPhail 1994) influence mate choice.

Hybridisation between benthic and limnetic species pairs is thought to be rare. McPhail (1994) estimates that approximately 1% of adults in both Paxton and Enos lakes are hybrids. The low frequency of mature hybrids can be explained by selection against hybrids in the wild. Although F1, F2 hybrids and backcrosses between the two forms show no evidence of developmental or sterility barriers, there is some evidence of reduced foraging success and growth rate of hybrids in the wild (Hatfield and Schluter 1996, 1999, Vamosi and Schluter 1999). In addition, Vamosi and Schluter (1999) found significantly reduced mating success in F1 hybrid males compared to limnetic morphs in semi-natural conditions. It is not yet clear if reduced hybrid fitness is driven purely by ecological selection or if genetic inferiority plays a part (Rundle and Whitlock 2001). Interestingly, Kraak *et al.* (2001) reported an increase in the rate of hybridisation in the Enos Lake species pair. The cause of this increase is currently unknown but may possibly be associated with changes in environmental conditions.

Benthic-limnetic stickleback species pairs were initially thought to be restricted to six lakes on islands in the Strait of Georgia, British Columbia, cases have since been

found in Alaska (Cresko and Baker 1996), Iceland and Norway (Olafsdottir Pers Comm., Arnott Pers Comm.) bringing the total to nine. More than 30 lakes of varying size and altitude in the Strait of Georgia, British Columbia contain single 'generalist' species of sticklebacks. Despite the thousands of apparently suitable lakes with seemingly similar ecological conditions throughout their holarctic distribution, the number of sympatric populations of benthic and limnetic sticklebacks is limited. Why species pairs have formed in some areas and not others is one of the most interesting questions of stickleback evolution.

Two possible models of speciation can explain the evolution of benthic-limnetic species pairs.

1. Sympatric Speciation
2. Allopatric Speciation: 'Double Invasion Hypothesis, McPhail 1992' – Initial colonisation of freshwater lakes by marine fish, followed by a period of isolation and divergence, and then a second invasion by the same species where divergence was completed in sympatry.

Molecular analyses testing these possible models are not conclusive. If speciation occurred in sympatry multiple times then the species pairs in each lake should share the most recent common ancestor. Alternatively if sympatric speciation occurred only once and subsequent colonisation of other lakes followed, then all benthic morphs should form a separate clade from limnetic morphs. These clades should be monophyletic while marine populations form outgroups. If allopatric speciation occurred following the 'double invasion hypothesis' it would be expected that limnetic and benthic morphs would be paraphyletic with marine populations.

Lack of reciprocal monophyly between benthics and limnetics across lakes suggests that limnetic and benthic species pairs have evolved independently at least 3 times (parallel evolution) rather than from one single divergence (Taylor and McPhail 2000). In two of the four Canadian lakes examined, limnetic and benthic morphs share recent common ancestry as predicted under a sympatric speciation model but morphs from the other two lakes are paraphyletic with marine populations. These results are difficult to interpret as recent common ancestry may also be explained by secondary invasion followed by introgression. Lineage sorting, the fixation of difference gene lineages from a common ancestral source provides further complexity to the interpretation of the tree topology. Additionally, the maximum likelihood phylogeny on which this analysis is based has low bootstrap support at many nodes.

Pairwise genetic distances calculated from microsatellite data indicate that limnetics from all four lakes were less divergent from pooled marine stickleback populations than benthic morphs (Taylor and McPhail 2000). This is consistent with the allopatric 'double invasion hypothesis' where the 'limnetics' were part of the second wave of colonisation. From these results, Taylor and McPhail (2000) support the double invasion hypothesis and suggest that the evolution of stickleback species pairs is not only contingent upon ecological selection as suggested by Schluter (1996), but also upon geological history (i.e. the ability of marine fish to invade freshwater lakes due to changes in sea level caused by glaciation).

ANADROMOUS AND FRESHWATER STICKLEBACKS

Anadromous and freshwater sticklebacks form a species pair noticeable by their divergent morphological and life history traits. Sticklebacks display three general lifestyles (purely marine, anadromous, and purely freshwater). Owing to their morphological similarity, the distinction between marine and anadromous sticklebacks is unclear. McPhail (1994) considers these forms to be part of the same breeding population, justifying his argument by their ability to reproduce in a variety of conditions. However, Baker (1994) argues that marine and anadromous fish are from distinct populations with selection pressures unique to each lifestyle (perhaps rightly so given the vast ecological divergence between estuarine and rocky shore habitats). The nature of the relationship between marine and anadromous forms is important because it has direct bearing on the evolution of anadromous and freshwater species pairs. This is one of the questions I address in my thesis.

At the start of the breeding season (typically May) anadromous sticklebacks migrate from the ocean into estuaries where they breed in sympatry with stream resident freshwater sticklebacks. Anadromous and freshwater sticklebacks differ in several morphological traits. Typically, anadromous fish are large, silver in colouration, have lateral plates, a tail keel, long, crenated dorsal and pelvic spines and numerous long, slim, gill rakers. In contrast, freshwater fish are smaller, have green-brown dorsal colouration, completely lack or possess only a low number of lateral plates, lack a tail keel, have shorter dorsal and pelvic spines, and fewer shorter gill rakers (Figure 1.1). Whilst these differences (especially plate morphology) are quite obvious, designation of fish to either the anadromous or

freshwater class based on these morphological traits must be performed with extreme caution (Hagen and Moodie 1982, Baumgartner 1995). Fish of varying degrees of intermediate phenotypes (likely to be hybrids and backcrosses) are sometimes found within the same habitat. If hybridisation is occurring, morphological classification may be unreliable as phenotype may not reflect the level of introgression in the genotype (e.g. Goodman *et al.* 1999). Despite the flaws in the morphological approach, most studies of sympatric anadromous-freshwater sticklebacks have used morphology as an indicator of life history status due to the difficultly of identifying an anadromous individual based on it's transient presence in the stream. The use of genetic markers to identify ancestry provides an alternative and more accurate approach, and studies of anadromous and freshwater sticklebacks utilising this method are sorely needed. Apart from obvious morphological and life history differences mentioned above, anadromous and freshwater sticklebacks also differ in behaviour (e.g. Mackney and Hughes 1995), physiology (Guderley 1995), and ecology (McPhail 1995). Withler and McPhail (1985) detected significant differences in allozyme frequencies between allopatric anadromous and freshwater populations sampled from British Columbia and similarly, Taylor and McPhail (2000) found significant differences in allele frequency at six microsatellite loci between allopatric marine and lake populations. McKinnon *et al.* (2004) showed that anadromous and freshwater morphs sampled sympatrically or from close allopatric populations were more genetically similar to each other than they were to their respective morphotypes sampled from other populations. Noticeably, in an analysis of sympatric anadromous and freshwater morphs in Lake Harutori, Japan, Higuchi and Goto (1996) did not detect significant differences in allele frequencies at 17 allozyme loci.

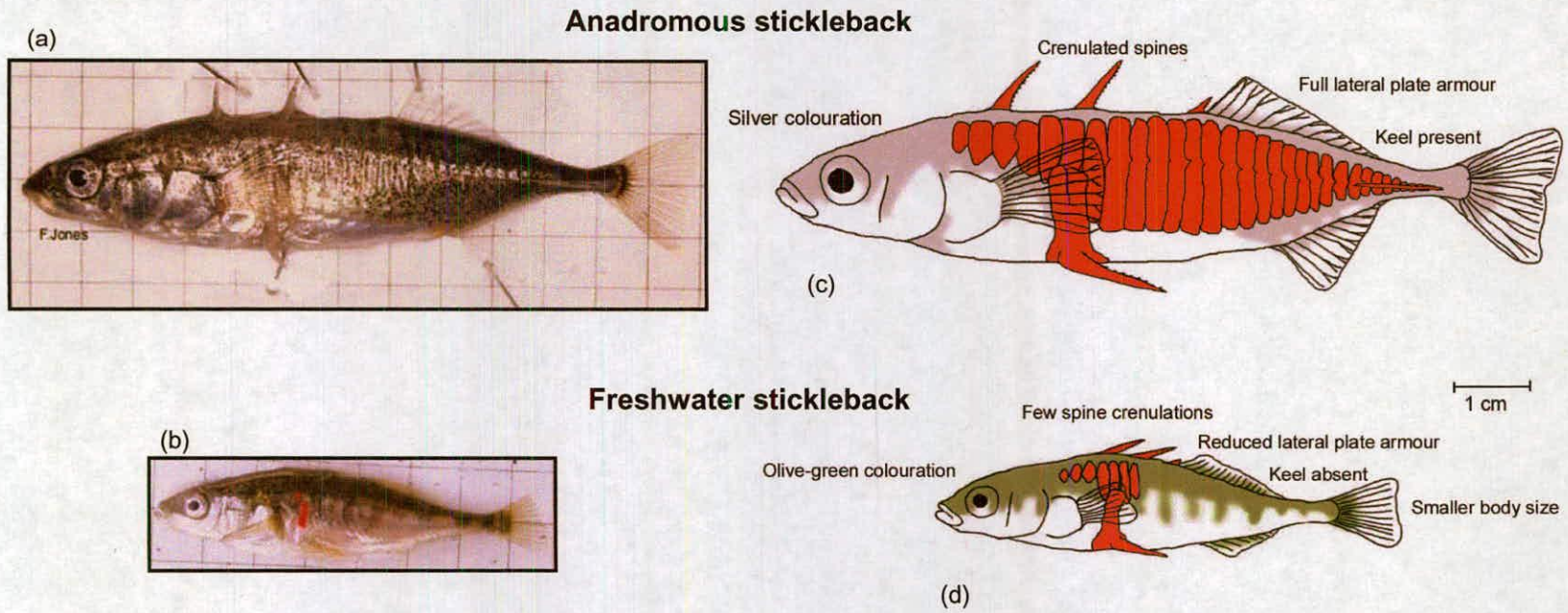


Figure 1.1. Photographs and line drawings of anadromous (a and c) and freshwater (b and d) female sticklebacks from the River Tyne, Scotland. Grid squares in photographs are 5mm. A red visible elastomer tag used for mark-recapture study can be seen under the pelvic girdle in (b). The pelvic girdle, lateral plates, spines and keel are coloured red in line drawings.

A substantial amount is known about the nature of divergence between anadromous and freshwater sticklebacks. The role of premating isolation in maintaining divergence in this species pair appears to differ throughout their distribution and this may indicate differing stages of speciation. However, this is difficult to conclude because of the differing sampling and experimental approaches used to determine assortative mate choice (e.g. compare Ziuganov 1995, Hay and McPhail 1975, McKinnon *et al.* 2004). In anadromous and freshwater morphs from the Little Campbell River, Canada, there is also evidence that differences in the timing of the breeding season as well as laboratory and observational field evidence of differences in microhabitat nesting preferences may contribute to premating isolation (Hagen 1967). Hybrid zones between anadromous and freshwater sticklebacks occur in lower reaches of rivers throughout their distribution (e.g. Hagen 1967, Hay and McPhail 2000) and appear to be relatively stable (Hay and McPhail 2000). Since premating isolation is not complete, this suggests that the fusing effects of hybridisation are balanced by either endogenous or exogenous selection against hybrids. There is little evidence of hybrid inferiority in laboratory conditions; hybrids are viable (Hagen 1967) and interfertile (McPhail 1994) thus it has been inferred that exogenous selection must play an important role (Hagen 1967, Moore 1977) in maintaining divergence. As yet, empirical evidence of selection against anadromous and freshwater hybrids does not exist.

A series of landmark studies have further highlighted the utility of sticklebacks for studies of the genetics of evolution (Peichel *et al.* 2000, Colosimo *et al.* 2004, Cresko *et al.* 2004, Shapiro *et al.* 2004). These studies have addressed three major questions concerning genetic architecture underlying morphological divergence.

Firstly, linkage mapping identified that much of the phenotypic diversity between limnetic and benthic and between anadromous and freshwater threespine sticklebacks could be explained by few QTL of large effect rather than many QTL of small effect (Piechel *et al.* 2001, Colosimo *et al.* 2004, Shapiro *et al.* 2004). Secondly, Shapiro *et al.* (2004) were able to identify the type of mutation that resulted in variation in pelvic morphology. By targeting genes homologous to pelvic reduction in mice, they determined that variation was not caused by substitutions in the coding region but rather by differential expression of the *Pitx 1* gene. Thirdly, simultaneous but independent studies by Cresko *et al.* (2004) and Colosimo *et al.* (2004) found that the same QTL of major effect underlie variation in the same traits in different stickleback populations. The existence of an EST and BAC library and the mapping of the stickleback genome will further improve the utility of the threespine stickleback for studies of speciation and evolution.

ANADROMOUS-FRESHWATER STICKLEBACK HYBRID ZONES

To date, the only study of reproductive isolation in a natural hybrid zone between anadromous and freshwater sticklebacks was carried out by Hagen (1967) in the Little Campbell River, Canada. He found evidence of differences in timing of breeding season and, in laboratory experiments performed on individuals collected hybrid zone, evidence of differences in microhabitat nesting preference but an absence of premating (behavioural) isolation. From his field studies, Hagen inferred that exogenous selection was acting against hybrids but was unable find evidence to support this in the wild population. His study may have lacked the power necessary to detect selection against hybrids due to the limited number of genetic markers available to infer ancestry. At present, empirical evidence of assortative mating

(premating isolation) and exogenous selection against hybrids (postmating isolation) in anadromous-freshwater species pairs *in situ* does not exist. However, recent improvements in the range of genetic tools available to researchers enables us to investigate these questions and this approach and line of questioning forms the basis of my thesis.

Like their North American counterparts, there is remarkable morphological diversity in Scottish stickleback populations (Campbell 1985, Giles 1983). Further, the geological history of Scotland in terms of the timing and cover of the last glacial maximum is similar to that of North America (Boulton *et al.* 1985). Aside from Hagen's (1967) study, detailed studies of anadromous-freshwater stickleback hybrid zones are completely lacking and the similarities between Scottish and North American sticklebacks provides a useful opportunity to investigate the generality of Hagen's inferences about reproductive isolation between anadromous-freshwater species pairs.

AIMS OF THESIS

Hagen's (1967) study of a hybrid zone in the Little Campbell River, Canada provided a wealth of information regarding the nature of reproductive isolation between anadromous and freshwater sticklebacks. However several key questions remain unanswered including whether assortative mating occurs in the wild and evidence for selection acting against hybrids. In this thesis, I set out to investigate the nature of both premating and postmating isolation in an anadromous-freshwater stickleback hybrid zone located in the River Tyne, Scotland, taking advantage of both theoretical developments in hybrid zone analyses and developments in genetic techniques.

In chapters 2 and 3, I explore evidence for premating isolation between anadromous and freshwater sticklebacks. In chapter 2, I specifically investigate the strength of assortative mating between sympatric morphotypes in controlled semi-natural conditions. This experimental design was conceived to improve upon previous laboratory-based studies of assortative mating by using 1) sympatric rather than allopatric populations, 2) allowing multiple matings rather than binomial choice, 3) allowing matings to occur rather than using behavioural indicators of mate choice, and, finally, 4) using genetics to assign parents to offspring and identify each mating *post-hoc*. In chapter 3, I investigate evidence for premating isolation between wild anadromous and freshwater sticklebacks using genetic analysis of samples collected from the field over time.

In chapter 4, I investigate the structure of the hybrid zone and the nature of selection acting on individuals by analysing clines in morphology, and allele frequencies. In

addition, I investigate hybrid fitness by resolving whether recombinant morphotypes and genotypes show reduced fitness at three different stages of ontogeny.

In chapter 5, I set out to determine whether QTL associated with traits in a Canadian stickleback population are associated with the same traits in Scottish stickleback populations. Using hybrid individuals as well as anadromous and freshwater individuals from the source populations, I investigate whether alleles at candidate loci explain both within and between species trait variation.

The chapters within this thesis are written in the style of separate papers which are intended for submission for publication. When viewed in context with the existing literature, it is hoped that these studies will provide a broader view of the nature of stickleback evolution throughout their distribution.

CHAPTER TWO

Lack of assortative mating between anadromous and freshwater sticklebacks from a hybrid zone.

ABSTRACT

In many sites throughout their distribution, anadromous and freshwater threespine stickleback (*Gasterosteus aculeatus)* morphs breed sympatrically, but the degree of reproductive isolation between the morphs appears to vary. Evidence suggests that premating isolation based on ecologically divergent traits (primarily size) is important in maintaining differences between the morphs. However, previous studies that have tested for assortative mating have typically used behavioural indicators of mate choice, which can be unreliable, rather than recording actual mating events. Further, earlier work investigating preferences in mate choice, collected morphs from two allopatric sites, rather than single sympatric sites or contact zones. This makes it difficult to draw reliable conclusions about the degree of assortative mating that may occur in sympatry. Here, we present a test for assortative mating based on morphotype and size, using anadromous and freshwater morphs collected from the same site in the River Tyne, Scotland. This experiment was carried out in controlled, semi-natural, conditions and mate choice was determined using genetic markers to assign parents to offspring *post hoc.* We found evidence for weak assortative mating based on morphotype by anadromous females, but not by freshwater females. In addition, freshwater females showed a preference to mate with large males, but, anadromous females' preference to mate with large males was negatively correlated with female body size. These results suggest that hybridisation between the two morphs is likely to occur readily in the River Tyne and that premating isolation does not play a large role in maintaining differences between anadromous and freshwater morphs. From these results we hypothesise that *in situ* hybridisation is likely to be biased towards matings between freshwater

females and anadromous males and that divergence between the two morphotypes is being maintained by ecologically dependent selection against hybrids.

INTRODUCTION

The threespine stickleback (*Gasterosteus aculeatus)* has undergone an adaptive radiation resulting in the parallel divergence of anadromous and freshwater morphs. These morphs vary in numerous traits including life history, morphology, behaviour, and genetic composition (McPhail 1994). Obvious divergent traits include body size and lateral plate number with anadromous morphs being larger and possessing complete sets of lateral plates compared to the smaller, low plated freshwater morphs (McPhail 1994). Genetic evidence suggests that freshwater morphs have arisen from the repeated and independent invasion of freshwater habitats by ancestral marine sticklebacks (McPhail 1994, McKinnon *et al.* 2004). At present, in many sites throughout their holarctic distribution, anadromous and freshwater morphs breed in sympatry, but the degree of reproductive isolation appears to vary (Ziuganov 1995, Hagen 1967). For example, Zuiganov (1995) collected high and low plated morphs from a single site in Lake Azabachije, Russia, and found complete premating isolation in a binomial choice experiment (although the sample sizes were small, N=6). In contrast, Hagen estimated hybrids to represent 46% of individuals sampled in the Little Campbell River, Campbell. Of particular interest to evolutionary biologists are the relative roles of premating or postmating isolation in maintaining differences between the two morphs.

There is little evidence for genetic incompatibilities between the two morphs. Laboratory crosses of plate morphs showed that F1 hybrids and backcrosses are viable, have equivalent mortality rates to crosses within morphotype (Hagen 1967), and are interfertile (McPhail 1994). Intermediate morphotypes are observed at low frequency in many sites (McPhail 1994, F. Jones Pers. Obs.). If postmating isolation exists, it is likely to be ecologically dependent (hybrid fitness is dependent upon ecological conditions in the wild), but at present evidence for this is indirect.

In contrast, several studies have investigated the role of premating isolation between anadromous and freshwater sticklebacks. Temporal differences in breeding season exist (Hagen 1967, McPhail 1994), but even in such populations the breeding seasons still overlap. Under laboratory conditions, different plate morphs from the Little Campbell River, Canada, vary in their microhabitat preference for nesting sites (Hagen 1967) and this is likely to contribute to premating isolation.

Sexual selection in the form of assortative mating (preference for mating with own type) is also likely to play a role in premating isolation, and many studies have investigated this possibility (Hagen 1967, Hay and McPhail 1975, McPhail and Hay 1983, Zuiganov 1995, Ishikawa and Mori 2000, Scott 2004, McKinnon *et al.* 2004), although experimental approaches have varied. Mating in sticklebacks is based primarily on female choice (McPhail 1994), although male choice may also occur (Rowland 2004). The presence of sexual dimorphism is indicative of sexual selection, and male sticklebacks display costly secondary sexual traits (Bakker and Milinski 1993, Hill 1999). Choosiness in females is rewarded by fitness benefits such as superior paternal care (McKinnon 1996), and good genes (e.g. Barber *et al.* 2001). Female sticklebacks show a preference for a number of different male traits

such as red colouration (McLennan and McPhail 1990, Milinski and Bakker 1990, Bakker and Milinski 1991, McKinnon 1995), blue eye colour (Bakker and Rowland 1995), optimal MHC alleles (Reusch *et al.* 2002) and larger body size (Rowland 1989). However, males may also be choosy and will court larger females when given a choice (Kraak and Bakker 1998).

Evidence suggests that assortative mate choice is based on ecologically divergent traits and is important in maintaining differences between the morphs (McKinnon *et al.* 2004). Nagel and Schluter (1998) found that body size plays a role in assortative mating because hybridisation between limnetic and benthic sticklebacks collected from different sites occurred only between the larger individuals of the smaller species and smaller individuals of the larger species. In a landmark study, McKinnon and colleagues (2004) were able to uncouple body size from other morphological traits by manipulating rearing conditions in the laboratory. They found that mating 'incompatibilities' between pairs of anadromous and freshwater sticklebacks were largely explained by differences in body size although preference for own morphotype still remained significant. These studies suggest that the difference in body size between anadromous and freshwater morphs in a sympatric population may affect the degree of assortative mating.

Previous studies of assortative mating between anadromous and freshwater sticklebacks have several weaknesses. Most studies used morphs collected from allopatric rather than sympatric populations (e.g. McPhail and Hay 1983, McKinnon *et al.* 2004, Scott 2004, Ishikawa and Mori 2000). By sampling either side of the contact zone, this approach avoids the problem of misidentification of morphotypes in a hybrid zone. However, it also introduces additional complications. Assortative

mating between fire-bellied toads was stronger outside than within the hybrid zone (Szymura and Barton 1986) and significant assortment between populations of the same subspecies have been found in grasshoppers (*Chorthippus parallelus,* Butlin 1998). It is possible that a similar effect occurs in sticklebacks. Females from each particular habitat have had no prior experience with the alternative morph or its habitat and prior experience is likely to have profound implications for mate choice (Breden *et al.* 1995, Magurran and Ramnarine 2004). In addition, differences in ecological variables such as water quality, microhabitat, food availability, and predation pressure could all substantially influence both female perception and male courtship (e.g. Houde and Endler 1990, Ward *et al.* 2004). Further, if reinforcement of mate preferences occurs, then assortative mating in sympatric populations may actually be stronger than assortative mating between allopatric populations. Reinforcement is a theory put forward to explain the observation of stronger mating discrimination between species living in sympatry than species living in allopatry (Dobzhansky 1951). This strengthening of mate discrimination is thought to have evolved in response to maladaptive hybridisation. If selection against hybrids in an anadromous freshwater stickleback hybrid zone occurs, then reinforcement of assortative mating might occur, however at present, evidence for reinforcement driving premating isolation is currently lacking. Thus, tests for assortative mating using allopatric populations of sticklebacks may not reflect mate choice in a contact zone.

Other drawbacks of some experiments include dichotomous choice designs (e.g. Hagen 1967, Hay and McPhail 1975, Zuiganov 1995, McKinnon 2004), where a female's preference is judged on her behaviour in front of two males. Results from these experiments may not reflect the true mating behaviour in sympatric sites

where many males are available at any given time. Finally, some experiments used behavioural indicators of mate choice such as female 'head up' posture (Hagen 1967 or nest inspection (McKinnon 2004). Again, these estimates may not be reliable indicators of mate choice. Conducting a study of assortative mating in a natural population, however, would also be difficult because the identity of the individuals present cannot be controlled. Ideally, in order to study premating isolation between divergent morphs researchers should utilise individuals collected from a site where they exist in sympatry, use successful mating events as a mate choice indicator, and allow females the choice of more than two males.

Here, we present a test for assortative mating using anadromous and freshwater sticklebacks collected from the same site in the River Tyne, Scotland. In this river, anadromous and freshwater sticklebacks differ considerably in a number of morphological traits and their breeding seasons overlap for more than three months (Chapter 3). Our primary aim was determine if hybridisation or assortative mating occur when anadromous and freshwater sticklebacks from the River Tyne breed in sympatry. We set out to address three specific questions: firstly, whether assortative mating occurs based on morphotype, secondly, whether the strength of assortative mating differs between morphotypes and finally, whether mate choice is influenced by standard length.

MATERIALS AND METHODS

SAMPLE COLLECTION AND EXPERIMENTAL DESIGN

In May 2002, adult sticklebacks were captured using wire mesh minnow traps from a 20m freshwater stretch of the River Tyne, East Lothian, Scotland (55"59.5'N 2"37.8'W). Based on morphological knowledge of upstream freshwater populations and downstream rockpool populations, fish were sorted using morphological criteria into (i) olive-green, small, low plated, keel absent 'freshwater' morphs, (ii) silver, high plated, large, keel present 'anadromous' morphs or (iii) 'hybrid' morphs with mixed combinations of these traits. Hybrid morphs were returned to the river at the site of capture. Under this classification, using Reimchen's (2000) description of lateral plate positions, low plated morphs possessed anterior lateral plates and sometimes a single posterior plate; high plated morphs possessed both anterior plates and a complete set of posterior lateral plates, and hybrid morphs possessed anterior plates and an incomplete set of posterior plates. Sixty-five of each of the freshwater and anadromous fish were transported to the University of Edinburgh and housed in the laboratory at 16 °C under a 12 hour light-dark regime. Ten or fewer fish of the same morphotype were housed per 40 litre tank, and fed on mixed diet of live daphnia (*Daphnia magna*) and frozen bloodworm (Chironomid larvae) twice daily.

After three weeks, 16 individuals of each sex and morphotype (64 fish total) were photographed against a 5mm grid and fin clipped for the purposes of individual identification using genetic markers. Standard length was measured from analysis of digital photographs. To ensure correct sex identification, these fish were chosen on the basis of their secondary sexual characteristics (gravidity of females, redness of chin and blue eye colouration of males). Individuals were then placed into one of

four artificial outdoor ponds with each pond containing four males and four females of both 'anadromous' and 'freshwater' morphotypes (16 fish total).

The artificial ponds were located at the University of Edinburgh and were constructed by subdividing an existing concrete-lined pond (10m long x 4m wide, previously used as an aquatic botanic garden and devoid of fish) into four separate ponds (3m long x 2m wide x 1m deep) using porous horticultural ground sheeting suspended from scaffolding. A gravel and sand substrate and two species of aquatic plant (Broad leaved pond weed, *Potamogeton* spp. and Horned pond weed, *Zannichellia* spp) were provided. In addition, the diet of fish in these ponds was supplemented with bloodworm on a weekly basis. One month after the adults were released into the ponds, 100 fry (less than 10mm size) from each pond were sampled at random using dipnets and killed using a UK Home Office Schedule 1 method. We estimated that 100 fry would be a sufficient sample size to detect mating events in each pond, since the 64 possible mate combinations were unlikely to occur in a period of one month.

GENOTYPING

DNA was extracted from finclips of adults and fry using a chelex extraction protocol (Walsh *et al.* 1991) with 0.2mg/mL proteinase K. Fish were genotyped at eight microsatellite loci (Table 2.1) using 10uL volume polymerase chain reactions containing 1mM dNTP's, 0.4uM of each primer, 0.4 units of BioLine *taq* polymerase, 1 x BioLine Buffer, 2.0uL of DNA and varying $MgCl_2$ concentrations (Table 2.1). Amplification cycles consisted of 2 min denaturation at 94 ºC, followed by 25 cycles of 30 sec at annealling temperature (Table 2.1), 1 min at 72 ºC and 45 sec at 90 ºC, and finished with 4 min extension at 72 ºC. PCR products were run with internal

size standard (GS500-Liz) on an ABI 3730 capillary sequencer. Resulting electropherogram data was then analysed using Genemapper software v3.0.

PARENTAGE ASSIGNMENT

We used the maximum likelihood algorithm of the PAPA software (Duschesne *et al.* 2002) for parentage assignment. Initially, we tested the power of our eight markers to correctly allocate offspring to parents by performing test simulations. Simulations involved a two step procedure. Firstly, 32 male and 32 female real parental genotypes were used to generate 400 pseudo-offspring genotypes. Then, pseudo-offspring were allocated to parents using the likelihood method. These two steps were iterated 5000 times. To obtain a conservative estimate of allocation success, it was assumed that all parental individuals were able to mate with each other (i.e. placed in a single pond). A transmission error of 0.02, with even distribution across all alleles, was factored into the simulations at both the production of pseudo-offspring step and the allocation of pseudo-offspring to parents step. The mean proportion of offspring assigned to the correct parents was 0.996 (± 0.003 SD) indicating a very high allocation success with the eight markers genotyped. Genotypes of the 100 fry and 16 potential parents from each pond were then used to allocate each offspring a male and female parent from that pond. We used these allocations as indicators of mating events between parental individuals and examined the mating combinations for evidence of morphotype- or size-based assortative mating.

Table 2.1. Details of loci used. Reference 1 = Peichel *et al.* 2000, 2 = Largiader *et al.* 1998, 3 = Taylor 1998. * Fluorescent label, [a] Annealing temperature (°C) and $MgCl_2$ concentration (mM) in parentheses. Allele range is given in basepairs.

LOCUS		Primer Sequence (~'* 5' - 3')	Product Size (bp)	PCR Conditions[a]	Reference
Microsatellite Loci					
STN26	F	* GTATCGAAGTCTGAAGGCCG	106 - 124	60 (0.5)	1
	R	GTACAGCATGTGGTCGATGG			
STN96	F	* ACACCTTCGGCTCCATATCC	218 - 278	58 (3.0)	1
	R	CGCAGCTCTCTGCTTTGC			
STN130	F	*TTCGGCTTATTTTCTTACCTGC	116 - 154	59 (0.5)	1
	R	ATGTTGTAGGCGAGGACAGGATG			
Gac 3133	F	*CGCCCAGTTCCTGAACTTAG	127 - 191	56 (2.0)	2
	R	CATGGTGGGCTGACTGAC			
Gac 4170	F	*GCCGAGCCACATAGAGA	102 - 150	55 (0.5)	2
	R	CCAATATAACAGCCGAGCAG			
Gac 1097	F	*AGGAACTCTCTTCTTCTCTG	90 - 144	55 (1.5)	2
	R	CCCGGGTTAGTCACT			
Gac 1125	F	*CATCACACCCAGCCTCTC	149 - 223	57 (2.0)	2
	R	CCTCCCTCCAACTCTTATCA			
Gac u7	F	* CAAAAGCAACAATCGACAAG	91 - 135	56 (2.0)	3
	R	CAATAACTGGAAGAGTGG			

STATISTICAL ANALYSES

Initially, we performed an ANOVA to test for significant differences in standard length between parental morphotypes placed in each of the ponds. This was done using standard length as a dependent variable, and pond, morphotype and sex as independent factors.

Then, using the parental assignments, we investigated female mating behaviour in several different ways. Firstly, for each female adult we calculated two variables: (i) the number of different fry sampled, and (ii) the number of different mating partners detected. We tested for differences in these variables between female morphotypes using t-tests.

Within each pool and overall, we then tested for evidence of morphotype-based assortative mating. We performed this analysis twice using different variables. Firstly, from the parental allocations, we calculated the number of distinct '*mating events*' (defined as unique combinations of female and male parents). Multiple fry allocated the same parents were assumed to have resulted from a single mating event. Mating events were then classified into one of the four possible parental morphotype combinations: anadromous mother–anadromous father, anadromous mother–freshwater father, freshwater mother–anadromous father, and freshwater mother–anadromous father. In each of the ponds, and pooling mating events across ponds, we tested whether the number of mating events involving male morphotypes was independent of female morphotype, using a G-test for independence with Williams's correction to reduce type I error (Sokal and Rohlf,1995). Then, we repeated the analysis using the total number of fry sampled from each of the parental morphotype combinations. Prior to this analysis, data were log transformed (0.001 was added to every cell, to avoid problems associated with logging zero values).

Next, we investigated mate choice at an individual level and tested for evidence of assortative mating based on morphotype or size. Because mating in sticklebacks is thought to be primarily based on female choice, we investigated mate choice from a female perspective. Firstly, we examined each female's preference for anadromous males by calculating the proportion of that individual's mating events that were with anadromous males. Here, we were interested in testing two distinct hypotheses: (1) whether the mean proportion of matings with anadromous males differed significantly from the null hypothesis of no preference for a particular male

morphotype (H_0: the proportion of matings with anadromous males = 0.5), and (2) whether the proportion of matings with anadromous males was associated with female morphotype and/or female standard length. We investigated the first hypothesis using t-tests and the second hypothesis using a mixed effects generalised linear model with the proportion of matings with anadromous males as the dependent variable, female morphotype and female standard length as a factor and covariate respectively and pond as a random factor.

Finally, we looked for evidence for size-based assortative mating by calculating, for each female, the average standard length of males mated and those not mated. We used paired t-tests to see if mated males were larger than unmated males. Then, we calculated the difference in standard length between mated and unmated males for each female. On this basis females mating with large males had a positive score and females mating with small males a negative score. We used size difference as a dependent variable in a linear mixed effects model to test for associations with female morphotype or female standard length. Female morphotype and female standard length were entered as a fixed factor and covariate respectively, and pond as a random factor.

Statistical tests were performed in SPlus software (v2000).

RESULTS

Of the 400 offspring genotyped, 372 (93%) were allocated parents, whilst 25 (6 %) were found to have ambiguous parents (two or more possible parental combinations with equal likelihood), and 3 (1%) were found to have no suitable parental combinations. 23 of the 25 individuals with ambiguous parental assignment were missing genotype data at either one or two of the eight loci and it is likely that these missing data were responsible for the lower assignment success than expected in the simulations. Genotyping errors are also likely to play a role since 3 fry did not match suitable parental combinations. Individuals with ambiguous or no parental allocation were excluded from further analysis.

The ANOVA of the standard length of parental individuals used in this pond experiment did not reveal any significant differences in the standard length of individuals in each of the ponds or any significant interactions between pond and sex or pond and morphotype (Table 2.2). We did detect a significant interaction between sex and morphotype (Table 2.2, Figure 2.1). A *post-hoc* test showed anadromous females were significantly larger than anadromous males (Fishers PLSD mean difference = 4.769, critical difference = 3.609, $p = 0.012$) but no significant difference in the standard length of freshwater females and males was detected (Fishers PLSD mean difference = -2.313, critical difference = 5.065, $p = 0.355$). Sex differences in size have been observed in other studies of anadromous and freshwater populations (Bell and Foster 1995).

Table 2.2. ANOVA table of standard length of adults used in pond experiment based on adjusted sums of squares (Type 3).

Variable	Df	F Ratio	P Value	
Pond	3	2.299	0.089	
Morphotype	1	31.581	0.000	***
Sex	1	0.664	0.419	
Pond*Morphotype	3	0.002	0.999	
Pond*Sex	3	1.076	0.368	
Morphotype*Sex	1	5.524	**0.023**	*
Pond*Morphotype*Sex	3	0.293	0.830	

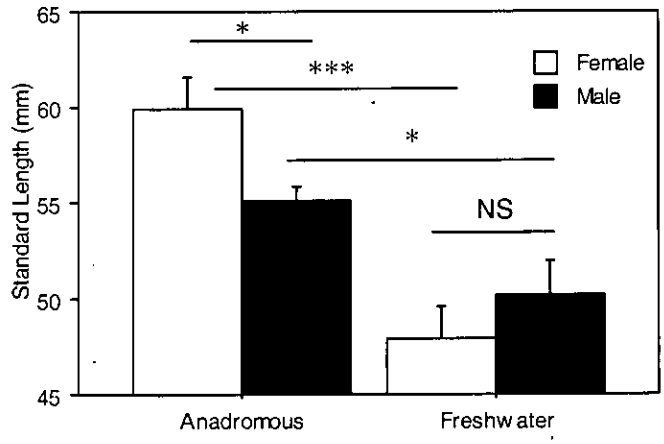


Figure 2.1. Mean standard length of anadromous and freshwater males and females. Error bars represent standard error. NS = not significant, * $p<0.05$, *** $p<0.001$

From examination of the parental allocations, we found that 41 of the 64 adults in the ponds were identified as a parent of at least one fry. The sex, morphotype and the pond location of the 13 adults not identified as likely parents, varied (Table 2.3). We sampled significantly more fry from anadromous females than from freshwater females (anadromous mean: 20.4, freshwater mean: 7.4, $t_{25}=3.310$, $p = 0.003$). In addition, females that did reproduce, mated on average with more than one male (mean = 2.7, range 1 – 5), and this differed significantly between female

morphotypes (anadromous mean = 3.3, freshwater mean = 2.2, $t_{25} = 2.466$, $p = 0.021$). Whilst anadromous females may be more promiscuous than freshwater females, this result is more likely to stem from our sampling methodology. As the number of fry sampled from a given female increases we are more likely to detect additional mates.

Table 2.3. The number, sex, and morphotype of adults not identified as parents in each pond.

Sex and Morphotype	Pond A	Pond B	Pond C	Pond D	Total
Female Freshwater	-	-	1	2	3
Female Anadromous	-	-	2	-	2
Male Freshwater	-	3	1	2	6
Male Anadromous	1	-	-	1	2
Total	1	3	4	5	13

Analysis of the number of mating events between adult morphotypes revealed no evidence for morphotype-based assortative mating (Table 2.4). Although we detected more mating events involving anadromous males than freshwater males, these mating events were independent of female morphotype. However, we did find evidence for non-random distribution of the number of fry among mating combinations in two of the ponds and overall (Table 2.5). In pond A, and overall, we sampled more fry from matings between anadromous females and anadromous males than we did from matings between freshwater females and anadromous

males. Conversely, in pond D, we sampled more fry from matings between freshwater females and freshwater males than we did from matings between freshwater females and anadromous males.

Anadromous females mated proportionally more with anadromous males than expected from the null hypothesis of an even proportion of matings with both male morphotypes (mean = 0.677, null hypothesis 0.5, $t_{12} = 2.579$, p = 0.024, Figure 2.2). This effect was not detected in freshwater females (mean = 0.667, null 0.5, $t_{13} = 1.654$, p = 0.122, Figure 2.2), although the small number of mating combinations meant that our power to test this deviation was low. A similar pattern was revealed in the analysis of the proportion of fry each individual female produced with anadromous males. Anadromous females produced proportionally more fry with anadromous males than expected under a null hypothesis of an even proportion to each male morphotype (mean = 0.723, null hypothesis = 0.5, $t_{12} = 3.123$, p = 0.009) but freshwater females did not (mean = 0.664, $t_{13} = 1.595$, p = 0.135, Figure 2.3). Anadromous and freshwater females did not differ significantly in the proportion of fry produced with anadromous males (Figure 2.3). In a linear mixed model, neither female morphotype, nor female standard length explained a significant variation in the proportion of matings with anadromous males (morphotype: $F_{1,20} = 0.218$, p = 0.645, see also Figure 2.2, standard length: $F_{1,20} = 1.557$, p = 0.227, Table 2.6). A large proportion of the total variation in the proportion of matings with anadromous males was explained by pond as a random factor (0.87).

Table 2.4. Number of Mating Events between each of the parental morphotypes.

POND	Anadromous Mother Anadromous Father	Anadromous Mother Freshwater Father	Freshwater Mother Anadromous Father	Freshwater Mother Freshwater Father	Adjusted G value	*df*	p value
A	8	3	8	7	0.986	1	0.321
B	8	6	2	1	0.087	1	0.767
C	7	1	5	2	0.566	1	0.452
D	5	7	0	2	1.767	1	0.184
Overall	28	17	15	12	0.305	1	0.581

Table 2.5. Number of fry sampled from each of the parental morphotypes combinations. * $p<0.05$, *** $p<0.001$.

POND	Anadromous Mother Anadromous Father	Anadromous Mother Freshwater Father	Freshwater Mother Anadromous Father	Freshwater Mother Freshwater Father	Adjusted G value	*df*	p value
A	52	8	25	11	3.982	1	0.046 *
B	53	15	15	9	3.167	1	0.075
C	60	7	27	2	0.251	1	0.617
D	36	35	0	17	20.636	1	<0.001 ***
Overall	201	65	67	39	5.837	1	0.016 *

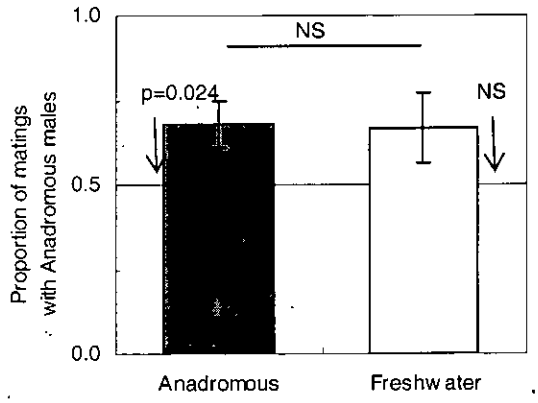


Figure 2.2. The proportion of matings with anadromous males grouped by female morphotype. Error bars represent standard error. NS= not significant. The proportion of matings with anadromous males was not significantly associated with female morphotype in a generalised linear model. In separate t-tests the proportion of matings with anadromous males was significantly greater than 0.5 for anadromous but not freshwater females.

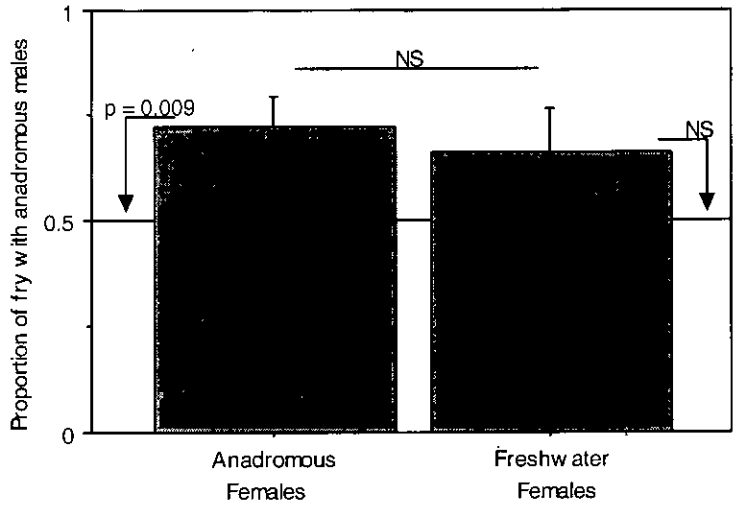


Figure 2.3. The mean proportion of individual females' fry that were that were allocated anadromous males as fathers. The proportion of fry with anadromous males was not significantly associated with female morphotype in a generalised linear model. In separate t-tests the proportion of fry with anadromous males was significantly greater than 0.5 for anadromous but not freshwater females.

Table 2.6. ANOVA table of linear mixed effects model of the individual proportion of matings with anadromous males. Pond entered as a random effect – variance component 1.64, residual variance = 0.23.

Variable	Estimate	SE	Df	F Ratio	P value
Intercept	7.01	3.82	1,20	1.409	0.249
Morphotype	-3.96	3.20	1,20	0.218	0.645
Standard Length	-0.10	0.06	1,20	1.557	0.227
Morphotype*Standard Length	0.05	0.05	1,20	1.144	0.298

We found evidence of size-assortative mating in which females of freshwater morphotype mated with large males (paired t-test: mean size of males mated = 54.001, mean size of males not mated 51.845, $t_{13} = 3.398$, $p = 0.004$) but this effect was not detected in anadromous morphotypes (paired t-test: mean size of males mated = 52.703, mean size of males not mated = 52.886, $t_{12} = -0.115$, $p = 0.910$) or among all females (paired t-test: mean size mated males = 53.376 , mean size males not mated = 52.347, $t_{26} = 1.217$, $p = 0.234$, Figure 2.4). We explored factors affecting the difference between mated and unmated males with a mixed model with female standard length and morphotype as fixed factors and pond as a random factor. We found a significant interaction between female standard length and female morphotype ($F_{1,20} = 5.400$, $p = 0.031$, Table 2.7). Freshwater females mated with large males independent of their own standard length, but a strong negative relationship between anadromous female standard length and difference in mated and unmated size was detected. Small anadromous females mated with larger males than did large anadromous females (Figure 2.5).

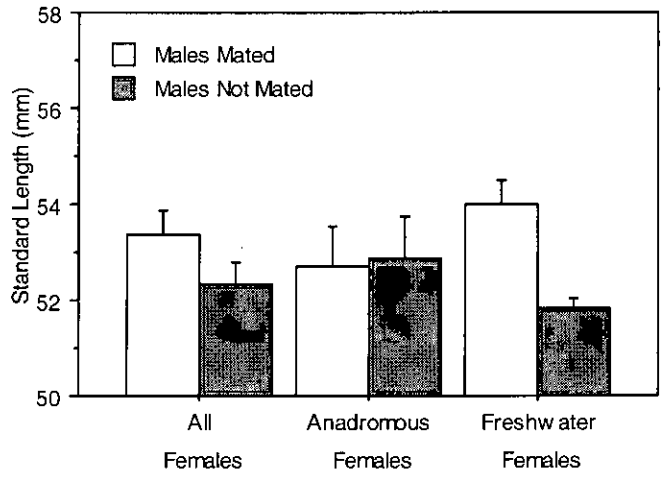


Figure 2.4. Mean standard length of males mated (white) and not mated (shaded), by all females, anadromous females, and freshwater females.

Table 2.7. ANOVA of mixed effects linear model of difference in size of mated and unmated males. Pond entered as a random effect – variance component 1.64, residual variance = 11.97.

Variable	Estimate	SE	Df	F Ratio	P value
Intercept	50.42	17.23	1,20	3.671	0.070
Female Morphotype	-42.78	18.16	1,20	0.162	0.692
Female Standard Length	-0.82	0.28	1,20	3.612	0.072
Morphotype*Standard Length	0.71	0.30	1,20	5.400	0.031

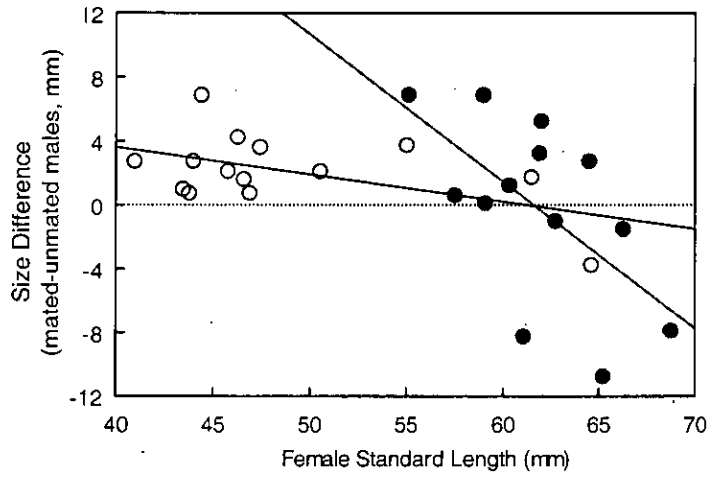


Figure 2.5. Difference in mated and unmated male standard length plotted against female standard length. Points above the dashed line represent females mating with large males, whilst points below the dashed line represent females mating with small males. Black points = anadromous females, white points = freshwater females.

DISCUSSION

Our results suggest that anadromous and freshwater morphs from the River Tyne, Scotland mate randomly with respect to morphotype when living in sympatry. In all four ponds, mating events were independent of morphotype. The lack of observed assortative mating explains the prevalence of intermediate morphotypes in the study site and is consistent with the findings of Hagen (1967) in his study of sticklebacks in the Little Campbell River (but see Hay and McPhail 1975). When viewed in combination with the fact that we have observed considerable overlap in anadromous and freshwater breeding seasons (Chapter 3), and caught both morphotypes in the same traps (regardless of microhabitat position, personal observation), these results suggest that premating isolation does not play a large

role in the maintenance of differences between anadromous and freshwater morphs in the River Tyne.

Our experiment offers several improvements over other previously published studies. Firstly, we used morphotypes collected from a single sympatric breeding site rather than two allopatric sites. Secondly, we used genetics to determine actual mating events rather than behavioural indicators of mate choice and, thirdly, by allowing females to mate with up to eight different males we improve on the no-choice or dichotomous choice designs of previous experiments. Further, we argue that, by conducting our experiment under semi-natural conditions, using divergent individuals who were sampled in sympatry, our experiment provides a more accurate reflection of the extent of premating isolation between anadromous and freshwater sticklebacks in the wild.

There is little evidence to suggest that courtship behaviour might differ between sympatric anadromous and freshwater male sticklebacks. In two sympatric populations of anadromous and freshwater sticklebacks, Hay and McPhail (2000) found no differences in courtship behaviour between male morphotypes. Owing to our experimental design, we were unable to investigate differences in female mating strategies such as clutch size, or differences male courtship, territoriality or paternal care, but this was not part of our aim. Rather, in these ponds, we hoped to reproduce controlled, semi natural conditions, to get a better idea of the type of mating that occurs in sympatric wild populations where factors such as territory defence, sneaky mating and paternal care are likely to influence reproductive isolation between anadromous and freshwater sticklebacks.

Our approach has three fundamental assumptions. Firstly, the assumption that our genetic markers provide us with enough power to correctly identify parents, secondly, that the classification of adults based on morphotype reflects their true genetic ancestry, and thirdly, that genetic incompatibilities or early exogenous selection does not influence the survival of hybrid eggs or fry. We are confident that our first assumption is true, as simulations using our eight markers show that more than 99% of parental allocations were correct. Our second assumption was that the classification of adults based on morphotype reflects their true genetic ancestry. This is an important issue to address because morphology can sometimes be an unreliable predictor of ancestral origin in a hybrid zone (e.g. Goodman *et al.* 1999). We aimed to reduce this error by using multiple morphological traits (number of lateral plates, presence of keel, colouration, and body size) rather than relying on a single trait to classify individuals. Using a separate data set of 392 adults of known genetic ancestry and morphology, we were able to test our power to assign individuals to the correct genetic group based on three of these morphological traits. We performed a discriminant function analysis using standard length, plate number (low, or complete), and keel presence/absence as discriminatory variables and found that individuals were assigned to the correct genetic group 92% of the time (F. Jones unpublished data). Misclassifications primarily involved genetically freshwater fish being classified as morphologically anadromous. The discriminant function analysis did not include the additional trait of colouration (because this data was unavailable) which is likely to further improve our discriminatory power. Therefore, the adult morphotypes we selected for this pond experiment are very likely to have the assumed anadromous and freshwater genetic ancestry. Our third assumption was that endogenous or exogenous factors did not influence the survival of hybrid eggs or fry. Hybrids from matings between anadromous and

freshwater morphs in the Little Campbell River did not show evidence of genetic incompatibilities or reduced viability (Hagen 1967). Given that we did not observe a deficit of fry from matings between morphotypes, we believe that it is unlikely that either endogenous or exogenous selection was stronger on these individuals. If this assumption was wrong, our measure of hybridisation (or matings between morphotypes) would be conservative, because selective pressures would act to remove hybrids before they were sampled.

On average, females mated with more than one partner during the one month period of this experiment and multiple matings were more frequent in anadromous females than freshwater females. Studies of a wild (anadromous) population, found the inter-clutch interval to range from 5 to 10.7 days (Boule and FitzGerald, 1989), and in laboratory studies of freshwater populations the inter-clutch interval was reported to range from 4-8 days Wootton (1974). The similar inter-clutch intervals of anadromous and freshwater sticklebacks suggests that the greater number of matings observed in anadromous females is more likely to arise from multiple paternity within clutches. A female's ability to mate with multiple males is likely to be affected by clutch size (and clutch size is greater in anadromous females, Hagen 1967) so it is possible that the multiple partners we detected in this experiment are a result of females depositing eggs in different males nests and therefore reflect active female choice. An alternative, but equally plausible explanation, is that multiple partners are a result of some males sneaking fertilisations after females deposit their eggs in the chosen male's nest. One female had five different mates but the number of fry sampled from each of these mates varied considerably (2-15). This variance would be expected in a situation where sneaky mating had occurred

(Largiader *et al.* 2001, Jones *et al.* 1998) but could also be explained by differences in the quality of paternal care or initial clutch size.

In each pond we sampled more fry from anadromous females and males than we did from freshwater females and males. This observation is best explained by the larger body size of anadromous females and males compared to freshwater morphotypes. In females, body size is positively associated with fecundity and anadromous females produce a greater number of eggs per clutch than freshwater females (Hagen 1967, Kraak and Bakker 1998). Large body size has been found to be advantageous in male territory defence and is likely to be correlated with the propensity to mate. During the reproductive period a male's territory needs to be defended from nest destruction, sneaky fertilisations, egg thievery, and predation of fry. In populations where there is large variance in male body size, large males defeat smaller males when competing directly for a territory (Rowland 1989). Goldschmidt *et al.* (1992) found that smaller inter-nest distance increased the probability of sneaky mating behaviour. In this experiment we stocked the ponds at a density of one male per 0.75 sq metre. This density is roughly equivalent to nest densities recorded in the wild (Whoriskey and FitzGerald 1994).

In two ponds, we detected significant heterogeneity in the number of fry sampled from each parental morphotype combination, despite the random distribution of mating events between morphotypes. In pond A and overall, proportionally more fry were sampled from anadromous males compared to freshwater males, but this bias was greater in anadromous females than in freshwater females. Pond B also showed a similar trend. In contrast, in pond D, whilst the proportion of fry sampled from anadromous mothers showed no bias toward male morphotype, amongst

freshwater mothers we observed a strong bias toward freshwater males (Table 2.5). These patterns are unlikely to result from the differences in body size between anadromous and freshwater morphotypes because we did not detect significant differences between ponds in the size of each adult morphotype present. Further, heterogeneity between ponds does not appear to result from the number or morphotype of adults who were not identified as parents (Table 2.3). Potential explanations for the observed patterns include the possibility that females may deposit different numbers of eggs with each male morphotype, or the success of male paternal care may differ depending on the morphotype of the mother.

On an individual level, our results revealed that anadromous females had a weak preference to mate with their own morphotype whilst freshwater females did not. This finding is consistent with a study of mating success between allopatric populations of Pacific anadromous and freshwater sticklebacks. Ishikawa and Mori (2000) found a higher courtship success rate when anadromous females were paired with their own morphotype than when paired with a freshwater male. They did not detect a significant difference in mating success when freshwater females were paired with anadromous or freshwater males. From these findings, it is possible to predict that, in the wild River Tyne population, hybridisation between freshwater females and anadromous males will occur at a higher rate than between anadromous females and freshwater males. We plan to investigate this possibility further by examining mitochondrial and nuclear markers in individuals collected from the hybrid zone (Chapter 3).

Our results also revealed an association between mating events and male body size such that both anadromous and freshwater females mated with large males more

often than small males. Freshwater females mated with large males independent of their own body size. This 'preference' for large males is consistent with studies conducted within stickleback populations of a single morphotype. Females prefer to mate with large males (Rowland 1989). But our results are inconsistent with studies of divergent stickleback populations where differences in body size were negatively associated with mate compatibility (Nagel and Schluter 1998, McKinnon *et al.* 2004). One possible explanation for this might be differential success by large and small males in obtaining and holding territories within each pond (Rowland 1983 a,b), since neither Nagel and Schluter (1998) or McKinnon *et al.* (2004) allowed for territory competition in their experimental designs. Further, in our experiment, we observed that small anadromous females mated with large males, whilst large anadromous females mated with small males. This may indicate that large females are actively choosing to mate with small males, but more likely, small males might be adopting a sneaky mating strategy, and using this strategy might be more successful in matings involving large females because of the larger clutch size (Hagen 1967, Kraak and Bakker 1998).

Using genetics to identify mating events, we found high levels of hybridisation between anadromous and freshwater sticklebacks collected from a contact zone. The direction of hybridisation in the wild is likely to be biased because freshwater females mate with large males and anadromous females mate proportionally more with their own morphotype. In the River Tyne population, premating isolation is unlikely to play a large role in maintaining divergence between anadromous and freshwater morphotypes. Future analyses should address two specific questions: Is hybridisation in the wild biased in any direction? And does selection against hybrids maintain divergence between anadromous and freshwater sticklebacks?

CHAPTER THREE

REPRODUCTIVE ISOLATION IN A THREESPINE STICKLEBACK (*Gasterosteus aculeatus*) HYBRID ZONE

ABSTRACT

In many estuarine sites, morphological differences between anadromous and freshwater threespine sticklebacks are being maintained despite breeding in sympatry. Here, we investigate the maintenance of this morphological divergence in a natural hybrid zone in the River Tyne, Scotland. We give a morphological and genetic description of the hybrid zone and using young of the year investigate the strength and nature of reproductive isolation between the morphs in the wild. In a previous study of anadromous and freshwater morphotypes from this system, we found weak assortative mating and predicted that hybridisation in the wild would show a directional bias towards mating between freshwater females and anadromous males. Using a Bayesian MCMC approach, we identified distinct anadromous and freshwater genetic clusters and the presence of hybrids in some sites of the River Tyne. Anadromous and freshwater sticklebacks differ significantly in shape, size and lateral plate number. Both morphological and genetic data revealed that anadromous and freshwater sticklebacks overlap spatially and temporally in the lower reaches of the river. In these sites, individuals of intermediate morphology and genetic ancestry are common and the presence of these hybrids indicates that reproductive isolation is incomplete. The significant heterozygote deficit in juveniles collected from sympatric sites is suggestive of assortative mating but might also be explained by selection against hybrid fry. This finding was supported by the existence of cytonuclear disequilibrium in homozygotes for anadromous and freshwater nuclear genes. However, the lack of cytonuclear disequilibrium in hybrids does not support our prediction of hybridisation being biased towards matings between freshwater females and anadromous males. We conclude

that hybridisation between anadromous and freshwater sticklebacks in the River Tyne is common and bi-directional, and that either assortative mating and/or early fry mortality contributes to reproductive isolation between the morphs. We discuss potential causes of the possible differences between mating patterns in the River Tyne, and River Tyne fish brought to a pond environment.

INTRODUCTION

In sites throughout the northern hemisphere migratory anadromous and resident freshwater threespine sticklebacks exist as species pairs: ecologically, morphologically, and genetically distinct populations that are in sympatry for at least some part of their life cycle (Taylor 1999). Despite breeding in sympatry in freshwater, these fish have undergone divergence in genetic and morphological traits associated with differences in ecology and life history (McPhail 1994, McKinnon 2002). Anadromous fish spend most of their lives at sea but migrate from the ocean to freshwater to breed. They nest in a variety of estuarine conditions including rock pools, intertidal reaches of rivers, and completely freshwater reaches of rivers. The upper limit of their migration into freshwater appears to be constrained by stream gradient (Hagen 1967) and in the lower reaches of rivers they often breed sympatrically with freshwater-resident sticklebacks. Morphs vary considerably in numerous morphological and meristic traits with freshwater resident morphs being smaller, possessing shorter spines, fewer gill rakers and lateral plates, than the more robust anadromous form (McPhail 1994, Walker and Bell 2000). Molecular evidence suggests that freshwater populations have arisen since the last ice

age (max. 20,000 years ago) by the repeated and independent invasion of freshwater habitats by marine sticklebacks (Taylor and McPhail 2000, McKinnon *et al.* 2004). The consistent loss of plates and many other morphological differences between the forms is therefore most likely a result of parallel evolution (McPhail 1994, McKinnon *et al.* 2001). The above-mentioned characteristics make sympatric populations of this species pair useful for studying factors influencing speciation and the parallel evolution of morphological traits.

The degree of premating isolation between anadromous and freshwater resident sticklebacks appears to vary throughout their distribution from complete premating isolation (Ziuganov 1995), to no premating isolation in laboratory conditions (Hagen 1967). Hagen's (1967) study of the Little Campbell River, Canada, was instrumental in showing that premating isolation might be affected by ecological factors. These factors are likely to include differences in the timing of breeding seasons, (Hagen 1967, McPhail 1995), and differences in microhabitat preference (Hagen 1967). In addition, sexual selection in the form of assortative mating may also contribute to premating isolation between anadromous and freshwater morphs (McKinnon *et al.* 2004, Boughman 2001). With the genetic tools available today, field studies of a hybrid zone can provide a more powerful approach for understanding the factors influencing reproductive isolation and maintenance of morphological differences between the members of this species pair.

In a previous study of anadromous and freshwater morphotypes collected from the River Tyne, Scotland, we showed that assortative mating is unlikely to be playing a

large role in maintaining divergence between anadromous and freshwater morphs (Chapter 2). In that experiment, we used divergent morphotypes collected from a single site, and found that hybridisation occurred readily. This result conflicted with studies of other anadromous and freshwater stickleback populations (Hay and McPhail 1975, Zuiganov 1995, Scott 2004, McKinnon 2004, but see Hagen 1967), but is more likely to reflect assortative mating in the wild because samples were collected from a single sympatric site, rather than two allopatric sites, and involved semi-natural conditions, rather than laboratory based binary choice experiments. Assortative mating experiments conducted in laboratory or semi-natural conditions may not reflect mating in the natural population because differences in the timing of breeding and microhabitat use might be affecting premating isolation. Intermediate morphs have been reported in many populations (e.g. Hagen, Hay and McPhail 2000), but despite the general belief that hybridisation is occurring, only one field based study of a hybrid zone has been reported (Hagen 1967). Using morphology and a single allozyme locus, Hagen estimated hybrids to represent approximately 21% of the overall population in the Little Campbell River. The lack of available genetic tools prevented him from investigating premating isolation in the wild, and he found no evidence of assortative mating in the laboratory.

The timing of the anadromous and freshwater stickleback breeding seasons differ throughout their distribution (McPhail, 1995). Resident freshwater fish breed throughout the summer, in some populations starting as early as March (Hagen 1967) and continuing until September (Baker 1994). There is greater variation in the timing of breeding in anadromous populations. This is largely dependent on the timing of the

anadromous run (McPhail 1994), which is strongly influenced by tides and lunar cycles. McPhail (1994) reported that anadromous fish on the west coast of Canada start to breed earlier than freshwater resident fish, but the breeding seasons still overlap considerably. In his study of anadromous and freshwater sticklebacks in the Little Campbell River, Canada, Hagen (1967) found a temporal overlap in breeding season for approximately two months. To date, we have no evidence of sympatric anadromous and freshwater morphs that show complete temporal isolation in breeding season. Therefore, differences in timing of breeding seasons alone are unlikely to cause complete premating isolation between these morphs.

Hagen (1967) presented evidence of differences in microhabitat preferences between anadromous and freshwater morphs from the Little Campbell River. In laboratory studies, he found significant differences in the nesting substrate and vegetation preferences of anadromous and freshwater males. He also described differences in microhabitat preference by plate morphs in Bonsall Creek, Canada, where high plated morphs are common in sandy substrate in the centre of the stream, and low plated morphs in the muddy substrate at the edge. It is unknown whether this corresponds to nesting location, and at present, no studies of nest location in a hybrid zone have been carried out. Nesting location may affect encounter rate by females and could contribute significantly to premating isolation between the morphs.

Here we describe the contact zone between anadromous and freshwater sticklebacks in the River Tyne, Scotland and set out to investigate the strength and nature of reproductive isolation between the morphs. Firstly, we developed a suite of genetic

markers to discriminate between anadromous and freshwater individuals, and used them to describe the morphological differences between anadromous and freshwater sticklebacks using geometric morphometric and traditional morphological analyses. This enabled us to examine the spatial and temporal distribution of fish in this river and identify hybrid/introgressed individuals. In sites where anadromous and freshwater sticklebacks breed sympatrically, we investigated reproductive isolation using genetic markers. From the results of our previous experiment (Chapter 2) we predicted that, in the natural population assortative mating would be weak, and that hybridisation may be biased towards matings between freshwater females and anadromous males. We investigated these predictions by testing for a heterozygote deficit in juveniles, which would arise if either non-random mating or selection against hybrid fry was occurring. In addition, we looked for evidence of directional hybridisation by performing tests for cytonuclear disequilibrium on hybrid individuals.

MATERIALS AND METHODS

FIELD WORK AND SAMPLE COLLECTION

Field work was conducted by Felicity Jones and Culum Brown at eight sites along the River Tyne, East Lothian, Scotland (grid reference at mouth of river 56"1.2'N 2" 34.1'W) during the years 2001-2003 (Figure 3.1). Site 1 was in rock pools at the mouth of the river, sites 2 and 3 were under tidal influence and 4 - 8 were freshwater sites. Several weirs were constructed across the river during the 19th century to assist neighbouring mills. The lowest weir, located between sites 4 and 5, contains a fish ladder to facilitate

the upstream movement of migratory fish. Other weirs exist between sites 5 and 6, and 7 and 8 and there is a waterfall between sites 6 and 7. All of these are likely to affect the movement of fauna within the river. In July 2001, July and September 2002, and then on a monthly basis from Jan 2003 – Dec 2003 sticklebacks were collected using wire mesh minnow traps immersed overnight. At each site, four traps were placed on each side of the river (eight in total) roughly 5m apart, equating to samples being collected from approximately a 20m stretch of river. Morphological measurements were taken, fish were fin-clipped for genetic analysis, photographed, tagged with visible elastomer tag (colour specific to the site), and released back into the river (Table 3.1). Tagging enabled us to identify previously sampled individuals, and to investigate changes in population size and the extent of within river movement (Jones *et al.* in prep). We also recorded the gravid status of each individual sampled. Young of the year collected in late September 2002, and adults from January - March 2003 were preserved in ethanol instead of being tagged and released. During July 2003 both adults and juveniles were caught in our traps. Adults were easily identified based on their large body size, and also in many cases due to their secondary sexual characteristics (red pigmentation in males, gravidity in females). Adults virtually disappeared from our samples in August 2003 (as a result of mortality or migration back out to sea). Juveniles less than 25mm standard length were not caught in our traps and therefore are not represented in our samples. Individuals sampled from September 2003 – December 2003, were used in genetic structure analysis (see below) but are not discussed elsewhere in this paper.

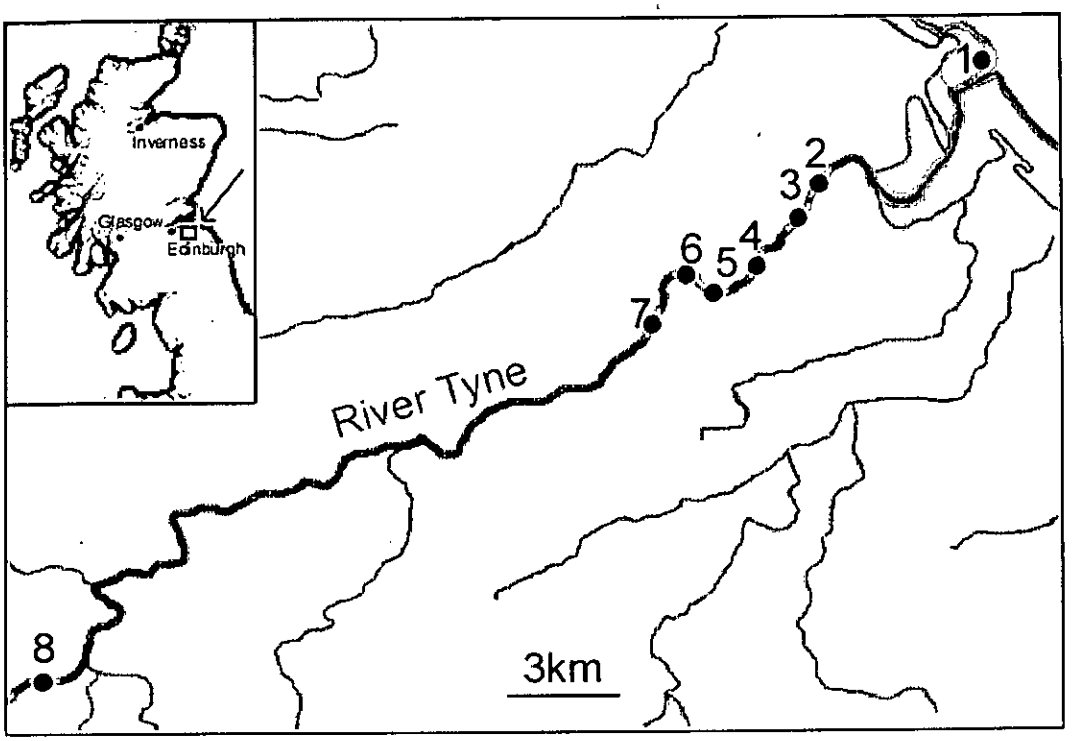


Figure 3.1. Map of sample sites (1-8) along River Tyne, Scotland.

Table 3.1. The number of individuals from which morphological data was collected for each site and month in 2003. Note, these samples sizes do not reflect the total number of individuals caught, but a random sample up to N = 57 of those individuals caught at each site in each month.

Site	2003														TOTAL
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Jul	Aug	Sep	Oct	Nov	Dec	
	Adults								Juveniles						
1	0	0	0	0	34	20	16	1	0	0	0	0	0	0	61
2	4	16	7	14	38	50	1	0	39	50	27	5	3	6	260
3	7	30	8	23	42	50	5	0	32	50	50	30	13	15	355
4	57	54	12	50	45	35	7	0	22	50	50	30	30	23	465
5	8	18	15	43	31	28	1	0	44	40	50	30	30	16	354
6	49	15	39	50	44	26	39	0	11	50	50	30	30	30	463
7	35	23	8	30	36	50	39	0	11	50	44	30	30	30	416
8	43	24	8	18	9	7	8	0	1	50	-	30	30	30	258
TOTAL	203	180	97	228	269	266	116	1	160	341	271	185	166	150	2632

MORPHOLOGY

Lateral plate counts of the left side of the body were performed in the field by gently prodding a sedated fish (MS222 anaesthetic in $NaHCO_3$ buffer) with a 'seeker' dissection tool. The length of 1st, 2nd dorsal and left pelvic spines was measured using callipers accurate to 0.1mm. Standard length of the fish was calculated from a digital image of the fish with reference to a background 5mm grid (live specimens), or measured using callipers (preserved specimens). Using the software tpsDig (Rohlf 2001) we recorded the x and y coordinates of eleven landmarks on the digital photographs (Figure 3.2). To avoid the gravidity of females affecting our analyses of shape, we used landmarks concentrated around the head region rather than the body. Landmark configurations were also used to calculate centroid size (defined as square root of the sum of squared distances of a set of landmarks from their centroid), which provided a useful measure of body size. In addition, we used tpsDig to measure four other traits: snout length, head depth, head length, and eye diameter.

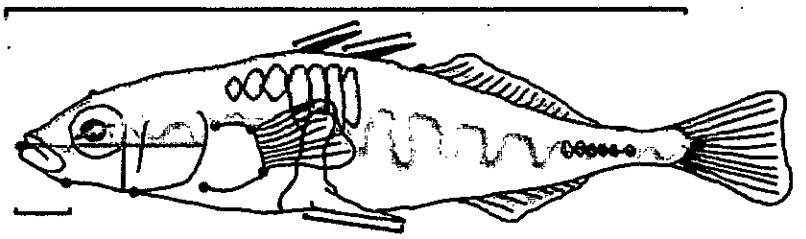


Figure 3.2. Morphological measurements taken from sticklebacks. Red points indicate landmarks for which x and y coordinates were digitised. Red lines indicate measurements of standard length, spine lengths, head depth, head length, eye diameter and jaw length. Lateral plate counts were also taken.

GENOTYPING

DNA extractions were performed on fin-clips using a chelex extraction protocol (Walsh *et al.* 1991) with 0.2mg/mL proteinase K. A suite of markers was chosen with the aim of discriminating between anadromous and freshwater morphs, and identifying hybrid/introgressed individuals. A total of 1961 fish from all eight sites (Table 3.2) were genotyped at 7 microsatellite loci, 1 mitochondrial single nucleotide polymorphism (SNP, located within the cytochrome b gene), and 3 nuclear SNPs positioned in introns of targeted genes (ATP1a2 intron 1, Myosin Heavy Chain intron 5, and beta Androgen Receptor intron 2, see Table 3.3 and details specified below). In addition, fish were sexed using labelled primers that amplify the 3' untranslated region of the Iso-citrate dehydrogenase (Idh) gene where a sex-linked insertion-deletion exists (Peichel K, unpublished data, see Table 3.3 and details specified below).

Table 3.2. Sample sizes of fish genotyped from each site each month. Age Class A = Adult, Age Class J = Juvenile.

Year		2001	2002			2003													Grand
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Site
Age class		A	A	J	J	A					A	J						J	Total
Site	1	19	-	-	-	-	34	20	16	1	**71**	-	-	-	-	-	-	**0**	**90**
	2	-	-	-	45	14	38	50	1	-	**103**	39	50	27	5	3	6	**130**	**278**
	3	-	-	-	45	23	42	50	5	-	**120**	32	50	50	30	13	15	**190**	**355**
	4	75	18	40	50	50	45	35	7	-	**137**	22	50	50	30	30	23	**205**	**525**
	5	-	-	-	45	-	-	-	-	-	**0**	-	-	50	-	-	-	**50**	**95**
	6	-	-	-	45	-	-	-	-	-	**0**	-	-	50	-	-	-	**50**	**95**
	7	51	21	6	45	30	36	50	39	-	**155**	11	50	44	30	30	30	**195**	**473**
	8	-	-	-	-	-	-	-	-	-	**0**	-	50	-	-	-	-	**50**	**50**
Total		**145**	**39**	**46**	**275**	**117**	**195**	**205**	**68**	**1**	**586**	**104**	**250**	**271**	**95**	**76**	**74**	**870**	**1961**

The mitochondrial SNP was identified using species-specific primers which amplified the cytochrome b gene. In addition, three nuclear SNPs were identified by designing primers for conserved exon regions spanning introns, using multiple alignments of sequences from multiple fish taxa. In some cases, primer design was aided by identifying candidate genes from the stickleback expressed sequence tagged (EST) database (Genbank) using the Blast search algorithm. Double stranded sequences of mitochondrial (1200bp) and nuclear introns (between 400 and 800bp) were obtained from 14 fish (7 from each of Site 1 and 8) and SNPs were identified.

A single diagnostic SNP was identified in the cytochrome b gene and fish from Sites 1 and 8 were discriminated by restriction fragment length polymorphism (RFLP) assay. Cytochrome b PCR products were amplified using forward and reverse primers labelled with different coloured fluorescent dyes. The enzyme *Hph I* was used to digest the PCR product at 37°C for 3 hours, producing two different coloured fragments in freshwater fish and 1 fragment in anadromous fish (Table 3.3).

At the three nuclear intron loci we identified both homozygous and heterozygous individuals for the alleles from analysis of sequence electropherograms. Since the detected SNPs could not be easily identified using a RFLP assay, we developed a novel screening assay. For each locus, this approach involved two separate PCRs, with a single reverse primer and one of two different forward primers. The forward primers were designed to amplify one of the two possible alleles by annealing immediately upstream of the SNP with the 3' base specific to one of the polymorphic nucleotides. The forward primers differed in length by one base pair at the 5' end, and

were labelled with different coloured fluorescent dyes (Table 3.3). Using positive and negative controls in the form of a heterozygous and two homozygous individuals for each of the alleles, which were identified by sequencing, we optimised the PCR conditions to high specificity (Table 3.3). This ensured non-specific amplification of the alternative allele did not occur. During screening, these three positive controls were also included in every PCR batch to enable identification of non-specific amplification, but we did not detect any.

For all loci, PCRs were performed in a 10uL volume containing 1mM dNTP's, 0.4uM of each primer, 0.4 units of BioLine *taq* polymerase, 1x BioLine Buffer, 2.0uL of DNA and with varying $MgCl_2$ concentrations (Table 3.3). Amplification cycles consisted of 2 min denaturation at 94°C, followed by 25 cycles of 30 sec at annealing temperature (Table 3.3), 1 min at 72°C and 45 sec at 90°C, and finished with 4 min extension at 72°C. Combined PCR products from all 12 loci were run with internal size standard (GS500-Liz) on an ABI 3730 capillary sequencer using a single capillary per individual. Resulting electropherograms were then analysed using Genemapper software v3.0. Details of sample sizes of fish genotyped from each site each month for the purposes of this study are shown in Table 3.2.

Table 3.3 Details of loci used. Reference 1 = Piechel *et al.* 2000, 2 = Jones *et al.* this study), 3 = Peichel (unpublished). FAM, VIC, NED, PET represent fluorescent labels, [a] 7bp 5' tail, [b] annealing temperature (°C) followed by $MgCl_2$ concentration (mM) in parentheses, [c] Primers redesigned using Genbank sequence for resizing purposes. Product sizes are given for freshwater (FW) and anadromous (AN) cytochrome b RFLP haplotypes, and for male specific (male) and all individuals (all) sex fragments. [d] 1bp length variation present.

LOCUS		Primer Sequence (~'* 5' - 3')	Product Size (bp)	PCR Conditions[b]	Reference
Microsatellite Loci					
STN9	F	PET GCGAAACGTTCATTTCAATTC	106 - 146	58 (2.0)	1[c]
	R	[a] AAAATTAATCGTTAGCACCCCTA			
STN26	F	NED GTATCGAAGTCTGAAGGCCG	106 - 126	60 (0.5)	1
	R	GTACAGCATGTGGTCGATGG			
STN94	F	PET GGCACGTCTCTCACTTTGAC	183 – 216	53 (1.5)	1[c]
	R	TNGATTTTACATTNTANCCTGGAC	[d]		
STN96	F	FAM ACACCTTCGGCTCCATATCC	218 - 280	58 (3.0)	1
	R	CGCAGCTCTCTGCTTTGC			
STN130	F	FAM TTCGGCTTATTTTCTTACCTGC	122 - 166	56 (0.5)	1[c]
	R	[a] ATGTTGTAGGCGAGGACAGGATG			
STN152	F	VIC ATGGAATATCGACAGAGCCG	228 - 346	57 (3.0)	1
	R	GTGCGGTCTGCTCATCAAGG			
STN208	F	VIC GAGTGGTTTCAAGCTGTGAGC	103 - 183	53 (1.5)	1
	R	CGCCTGTTCTTTACAAAGCC			
Nuclear Intron SNP's					
ATP1a2	F	PET TCTAAAAAATCTTTGTCCAACCC	79	60 (0.5)	2
	F	NED ATCTAAAAAATCTTTGTCCAACCA	80	62 (1.5)	
	R	GACCTGGGAGACGAAGAGTAAA			
BAR2	F	FAM AACATTACGGCATATTTTGTACTAAC	184	59 (0.5)	2
	F	VIC CAACATTACGGCATATTTTGTACTAAT	185	59 (0.5)	
	R	TGCGAAGTTATCATCCCTAAAGA			
Myo3-1HC	F	VIC TGAAGGTGTATCATCTGCTAATTTT	90	60 (0.5)	2
	F	FAM TTGAAGGTGTATCATCTGCTAATTTG	91	59 (0.5)	
	R	TGGATGACTCTTTTGGTGTTGA			
Mitochondrial SNP					
Cyt-b	F	FAM CCCTCCTTGGACTTTGCTTA	157, 326 (FW)	57 (1.5)	2
	R	NED TGAACAAGTGTGGCACCAG	483 (AN), 226, 257 (AN)		
Sex Marker					
Idh 3'UTR	F	GGGACGAGCAAGATTTATTGG	270 (male), 300 (all)	59 (1.5)	3
	R	PET TATCGTTAGCCAGGAGATGG			

STATISTICAL ANALYSIS

Morphology - We examined the differences in morphology between adult fish collected from Site 1 (rock pools) and Site 8 (upstream) in April – July 2003. To investigate differences in overall body shape we used a geometric morphometric approach. After performing a generalised procrustes alignment of landmark configurations to remove non-shape variation (for example, due to the position, orientation and size of the fish), we extracted partial warp scores from the remaining variation in landmark configurations. These shape variables were then entered as dependent variables in a MANOVA with sex and site as independent factors. Visualisations of shape differences were achieved using thin-plate splines to map the deformation in shape from Site 1 (anadromous) to Site 8 (freshwater) fish. To compare differences in specific morphological and meristic traits we performed t-tests comparing individuals of each sex from Sites 1 and 8 separately. Because we found significant differences in size, as indicated by both standard length and centroid size, statistical tests were performed on size adjusted trait scores (residuals from a regression of the trait against standard length). Finally, using morphometric, morphological (e.g. head length and depth) and meristic measurements (e.g. lateral plate number) collected from adult fish in all sites, we examined the distribution of morphotypes in the river and looked for evidence of morphotypes existing in sympatry. This analysis involved two steps. Firstly, a discriminant function analysis was performed to discriminate between fish from Site 1 and 8, and coefficients for canonical variables were extracted. These coefficients were then used to calculate the canonical score for each individual collected from all eight sites in the river. In this way, the morphology of fish from sites 2-7 is scored in terms of similarity to anadromous fish from Site 1 or freshwater fish from Site 8. We examined

the distribution of canonical scores within each site for evidence of geographic overlap of morphotypes.

Genetic Structure Analysis - The presence of (a) distinct genetic clusters in our entire data set of nuclear genotypes (1961 individuals, Table 3.3), and (b) within-site structuring, was tested using STRUCTURE v2.0 (Falush *et al.* 2003). This analysis utilised the model-based clustering algorithm of Pritchard *et al.* (2000) to cluster individuals based on their multilocus nuclear genotypes and accounted for the correlations between linked loci that arise in admixed populations (Falush *et al.* 2003). One of the big advantages of this approach is that it is possible to test for distinct genetic clusters without having any *a priori* assumptions about an individual's population of origin. For both analyses we estimated the posterior probabilities of there being K distinct genetic clusters within our data sets, where K=[1-8], and K=[1-3] for analysis (a) and (b) respectively. We tested for up to eight clusters in analysis (a) to incorporate the possibility of there being genetic distinctions between fish in each of the eight sites. This was done assuming an uniform prior for K, was repeated 3 times to assess convergence of $\mathrm{Ln}P(X|K)$, and involved a burn-in period of 100,000 replicates followed by 1,000,000 replicates for each run. The number of clusters was determined by the value of K with largest posterior probability following the guidelines of Pritchard *et al.* (2000). In the case of analysis (b), if two genetically distinct clusters existed in sympatry in a single site, then we would expect two genetic clusters (i.e. K to equal 2), and we call such sites 'sympatric sites'. Alternatively, if samples collected from a single site were from a randomly mating population we would expect K to equal 1.

Since we found K = 2 in our analysis of the entire data set (see results), we were able to assign individuals to distinct genetic clusters and identify putative hybrids using the individual ancestry assignment scores (*q*) obtained from the above analysis. These scores correspond to the probability of an individual having ancestry in one of the two putative source populations. We examined the spatial and age class distribution of ancestry scores in the river by plotting histograms of scores in each of the eight sites for adults (Apr – Jul) and juveniles (Jul – Dec) separately. Finally, 90% confidence intervals around these estimates were used to classify individuals as either "freshwater" (confidence intervals around *q* incorporate 0 but not 1), "hybrid" (confidence intervals around *q* incorporate 0.5 but not 0 or 1), "anadromous" (confidence intervals around *q* incorporate 1 but not 0). These classifications were used as indicators of an individual's nuclear genetic composition in analyses of female gravidity and cytonuclear disequilibrium.

Using gravidity of females as an indicator of reproductive condition, we were able to determine the temporal overlap in breeding season of the genetic groups. We calculated the proportion of genetically anadromous, hybrid and freshwater females that were gravid in each month from Sites 1-4 and Site 7. Females from sites of sympatry (Sites 2-4) were pooled.

Tests for reproductive isolation - Having determined that anadromous and freshwater sticklebacks exist in sympatry in sites 2-4, and additionally, that genetically hybrid individuals and individuals of intermediate morphotypes were present in these sites (see results), we investigated the strength and direction, if any, of premating isolation.

We used the genotypes of juveniles collected in July and August to determine the mating patterns of the adult breeding population. We chose not to include young of the year individuals sampled from September – December to exclude any possible effects of the anadromous migration out to sea and of selection against young of the year, on genotype frequencies. Firstly, we looked for evidence of non-random mating in sites 1-4, 7 and 8 by testing for departures from Hardy Weinberg Equilibrium. Specifically, we tested for heterozygote deficiency using the program FSTAT (version 2.9.3.2, Goudet, J. 2002). Significance was assessed by randomisation tests (where alleles were randomised among individuals within samples 50,000 times). Because our sample did not include fry <25mm we are unable to exclude the possible contribution of postmating selection against hybrid fry towards a heterozygote deficit.

Next, we investigated the nature of reproductive isolation and whether hybridisation was directional (where one combination of parental morphotypes hybridises more often than the other) using tests for cytonuclear disequilibrium. We tested for associations between maternally inherited mitochondrial genotypes and nuclear genotypes using the program CNDm (Basten and Asmussen 1997). In these tests, significant disequilibrium indicates non-random association between the nuclear and mitochondrial genotypes. Detection of significant cytonuclear disequilibrium within the anadromous and freshwater nuclear genotypes might arise from either assortative mating, or post-mating selection against fry, and significant cytonuclear disequilibrium within the heterozygous or hybrid nuclear genotype may provide evidence of directional hybridisation. Cytonuclear disequilibrium was tested using the nuclear genetic ancestry coefficient, q, estimated from the multilocus assignment test employed in STRUCTURE

(see above), and mitochondrial haplotypes in pooled juveniles collected from Sites 2-4 in the months of July and August. Tests were performed following the guidelines of Basten and Asmussen (1997). Significance of overall departures from random genotypic associations was assessed using Monte Carlo Markov Chain randomisations involving 100 batches of 1,000 observations whilst the significance of individual disequilibria was assessed using Fisher's exact tests.

RESULTS

We sampled a total of 2632 sticklebacks from the River Tyne during our field work in 2003 (Table 3.1). Sticklebacks were present in the rock pools at Site 1 in the months of May – Aug but were absent at all other times of the year. Based on these observations we believe that the anadromous sticklebacks migrate from the ocean to their breeding grounds between April and May. At Site 1, sticklebacks were more abundant, but not exclusive to, rock pools located above the mean high tide mark. These pools experience less tidal disturbance and contain fewer marine snails compared to lower pools (Pers. Obs.). In the lower reaches of the river, we caught both anadromous and freshwater morphotypes in the same trap.

Morphology

We found a significant difference in the shape of adult fish sampled from Sites 1 and 8, and significant differences in shape between sexes (MANOVA: Site $F_{54,221.3}=7.383$, $p<0.0001$, sex $F_{34,150}=4.757$, $p<0.0001$). In addition, we found a significant interaction between sex and site suggesting that the extent of sexual dimorphism in shape differs

in each site (sex*site $F_{16,76}$=2.394, p<0.0059). Consistent with other morphological descriptions of these fish (e.g. Walker and Bell 2000), anadromous fish have a more robust head shape than freshwater fish (Figure 3.3). In both sexes, fish from Site 1 were significantly larger (centroid size) and longer (standard length) than fish from Site 8 (Table 3.4). We found females from Site 1 to have significantly longer dorsal and pelvic spines and deeper heads than females from site 8, after adjusting for standard length. In contrast, males from Sites 1 and 8 did not differ significantly in these size-adjusted traits. Both males and females from Site 1 possessed significantly more lateral plates than males and females from Site 8. Examination of the distribution of canonical scores within each of the eight sites (Figure 3.4a), revealed that both anadromous and freshwater morphotypes were sampled from Sites 2-4, and that fish from Sites 5-7 are of freshwater morphotype only. Because lateral plate number as a canonical variable contributed a large amount of weighting in the canonical function we plotted the distribution of lateral plate morphotypes in each of the sites (Figure 3.4b). We also observed the presence of individuals of intermediate morphology in Sites 2 – 4 (Figure 3.4a,b).

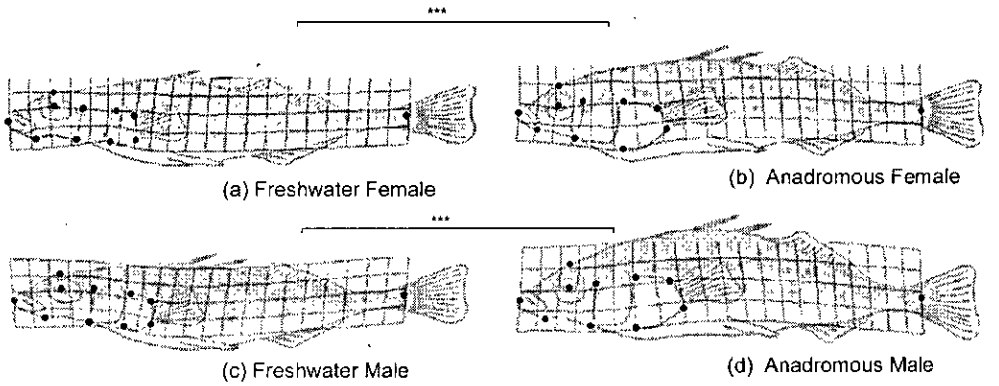


Figure 3.3. Visualisation of shape differences between anadromous and freshwater fish. For each sex, grids show the deformation in shape of an average consensus configuration into freshwater configuration (a) and (c) and anadromous configuration (b) and (d). Deformations have been magnified 3X to emphasise the shape differences. The background represents superimposition of a fish graphic into the estimated landmark configurations, but only changes in the position of the landmarks should be considered. *** represents significant differences in a MANOVA of partial warp scores at the $p<0.0001$ level.

Table 3.4. Morphological differences between anadromous (Site 1) and freshwater (Site 8) sticklebacks from the River Tyne. Measurements of spine lengths, and lateral plate counts were collected in the field, using callipers accurate to 0.01mm. All other measures were calculated from digital photographs. Measurements for all traits are in millimetres, except lateral plates which is a count, and centroid size which is calculated as the square root of the sum of squared distances of a set of landmarks from their centroid. For each sex, values in table represent mean trait values but statistical tests (t-tests) were performed on residuals from a regression of the trait against standard length to examine differences in size adjusted traits between fish from site 1 and site 8. SE represents standard error. NS $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ‡ Tests which remain significant after sequential Bonferroni correction (Sokal and Rohlf 1995).

Site	Sex	N	Standard Length			1st Dorsal Spine			2nd Dorsal Spine			Pelvic Spine			No. Lateral Plates		
			Mean	SE		Mean	SE		Mean	SE		Mean	SE		Mean	SE	
1	Females	33	61.211	0.63	‡	5.491	0.09		5.947	0.09	‡	9.240	0.13	‡	27.848	0.19	‡
8		22	47.029	1.56	***	3.727	0.13	*	4.070	0.14	**	6.000	0.20	**	4.682	0.18	***
1	Males	49	53.850	0.62	‡	5.235	0.09	NS	5.363	0.10	NS	8.571	0.11	NS	27.408	0.19	‡
8		27	40.502	0.70	***	3.672	0.12		4.063	0.08		5.539	0.11		5.593	0.28	***

Site	Sex	N	Eye Diameter			Head Depth			Head Length			Snout Length			Centroid Size		
			Mean	SE		Mean	SE		Mean	SE		Mean	SE		Mean	SE	
1	Females	13	4.595	0.06	NS	10.292	0.08	‡	16.333	0.17	NS	4.575	0.06	NS	54.720	0.70	‡
8		22	3.555	0.08		7.457	0.21	**	12.398	0.34		3.547	0.10		40.748	1.36	***
1	Males	33	4.528	0.04	NS	9.978	0.13	NS	16.569	0.19	NS	5.041	0.10	NS	48.102	0.53	‡
8		27	3.587	0.07		6.836	0.14		11.935	0.27		3.389	0.10		34.945	0.61	***

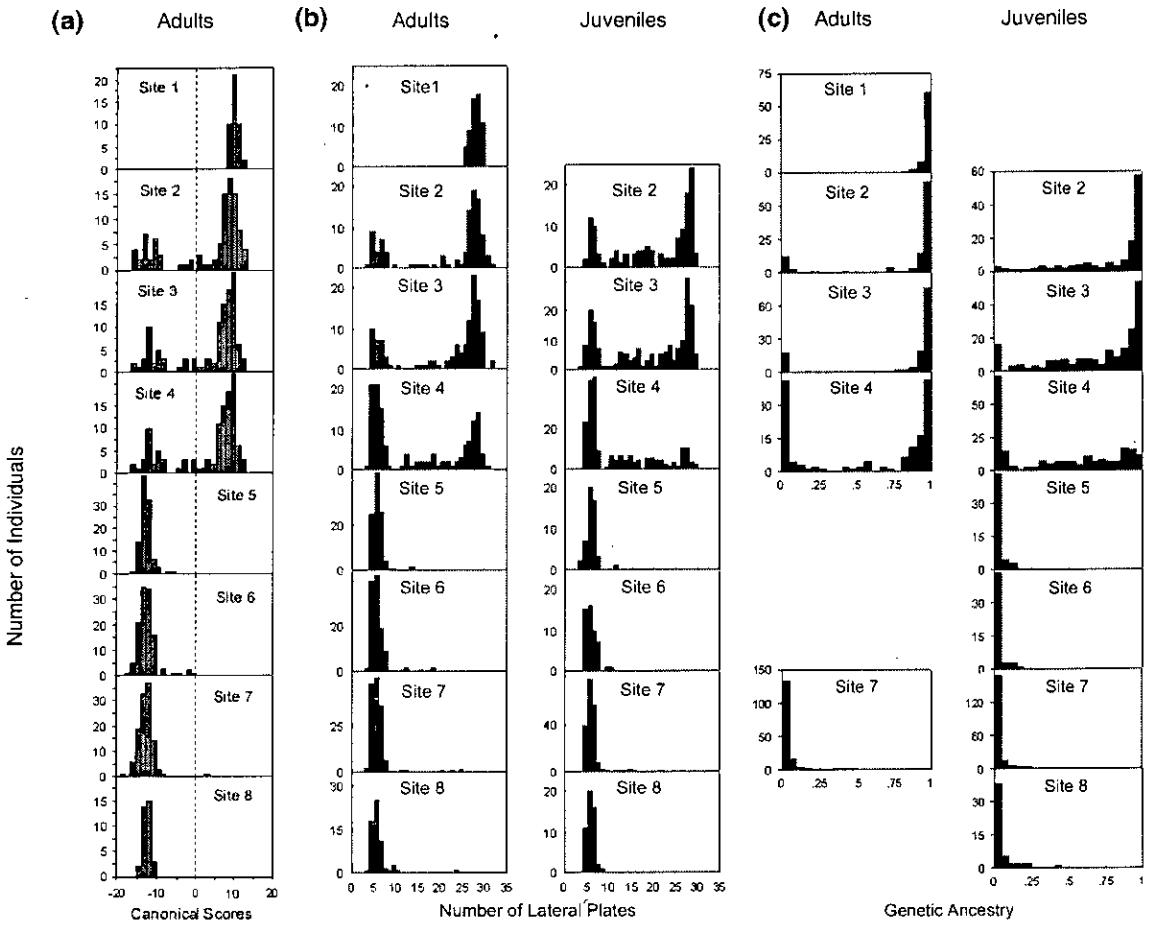


Figure 3.4. Distribution of (a) canonical scores based on morphology in Sites 1-8 for Adults (April – June 2003), (b) lateral plates in Adults (April –June 2003) and Juveniles (July – December 2003) and (c) genetic ancestry in Sites 1 – 8, for Adults (April – June 2003) and Juveniles (July – December 2003). Individuals with positive and negative canonical scores are of anadromous and freshwater morphology respectively. A genetic ancestry score of 0 represents freshwater and a genetic ancestry score of 1 represents anadromous ancestry. Lateral plate number (b) was used as one of the morphological traits in the canonical analysis (a) and had the heaviest weighting towards canonical scores. Genetic analysis of adult samples from Sites 5, 6, and 8 was not performed. No juveniles were collected from Site 1.

Genetic Structure

In total, we detected three different mitochondrial haplotypes that proved to be completely diagnostic between anadromous fish from Site 1 and freshwater fish from Site 8. Only haplotypes 1 and 2 were found in fish from Site 1, haplotype 2 being very rare (the frequency at Site 1 was 2%, which over all individuals equals 0.001%), haplotype 3 was found in 100% of fish from Site 8. None of the nuclear markers were completely diagnostic between fish from Site 1 and Site 8. At the three nuclear intron loci (MyoHC, bAR2 and ATP1a2), the frequency of "anadromous" alleles was 80%, 90% and 100% in Site 1, and 41%, 0%, and 77% in Site 8 respectively. Microsatellite loci showed considerable overlap in allele size between Site 1 and Site 8, although significant differences in allele frequency were detected at all loci (not shown).

STRUCTURE analysis of the entire data set (analysis a) revealed the most likely number of distinct genetic clusters in the River Tyne to be two (Figure 3.5, Appendix Tables 6 and 7). 97% of fish sampled from Site 1 were assigned to cluster 1, whilst 95% of fish sampled from site 8 were assigned to cluster 2. This, along with significant differences in allele frequency between sites 1 and 8, is indicative that anadromous and freshwater fish are of distinct gene pools. Analysis of genetic structure within each of the eight sites (analysis b) revealed the most likely number of genetic clusters to be two in Sites 1–4, but only one in Sites 5-8 (Table 3.5). These results suggest that anadromous and freshwater morphs exist in sympatry in each of the lower four sites. Examination of the distribution of q in each of the sites provides further support for the existence of both genetically anadromous and freshwater fish in Sites 2-4 but does not uphold the presence of genetically distinct individuals in Site 1 (see Figure 3.4c). These

conflicting results for Site 1 might be best explained by a spurious result from the within-site genetic cluster analysis due to the small sample size (N=90). We observed much higher allelic diversity in anadromous fish (Appendix Tables 2 and 3). This, coupled with a small sample size, would reduce the power of a cluster analysis based on multilocus genotypes. Our data indicate that anadromous and freshwater sticklebacks are both genetically and morphologically distinct but overlap spatially in the lower reaches of the River Tyne. We conclude, from this analysis that Sites 2-4 represent sites of spatial overlap of anadromous and freshwater sticklebacks and call these 'sympatric sites'.

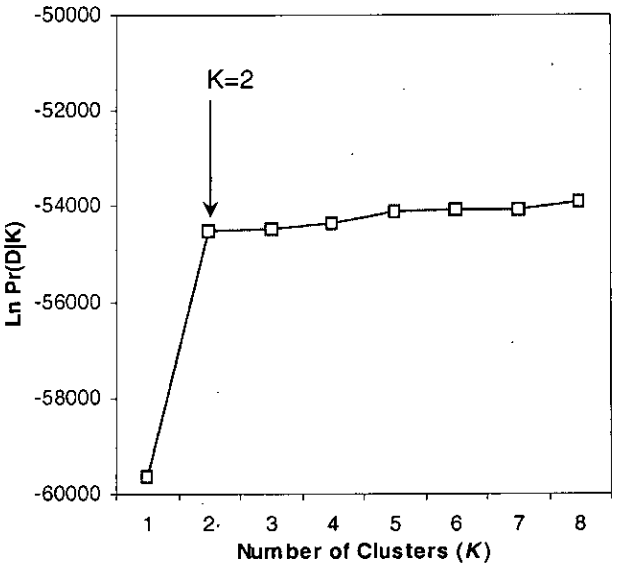


Figure 3.5. The Ln probability of there being *K* genetic clusters in the entire data set of 1961 individuals. Following the guidelines of Pritchard and Donelly (2000), the most likely value of K is interpreted to be the lowest value of K at the start of the plateau (in this case, K=2).

Table 3.5. (continued next page). Ln likelihood values of there being K=[1-3] genetic clusters within samples collected from each of Sites 1-8, and the proportion of individuals assigned to each of the 1-3 clusters. Following the guidelines of Pritchard, Stephens and Donelly (2001), the most likely value of K is interpreted to be the smallest value of K at the start of the plateau. The most likely value of K has been symbolised with * in each case. See also Appendix Table 8 for more information.

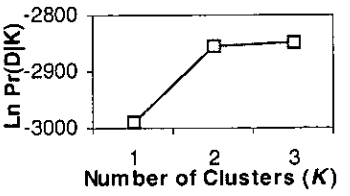
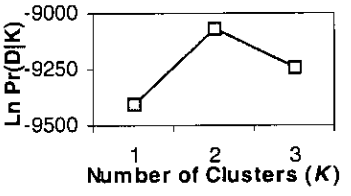
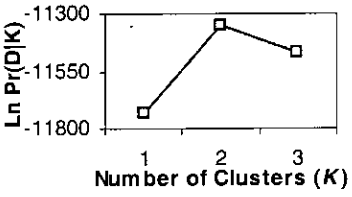
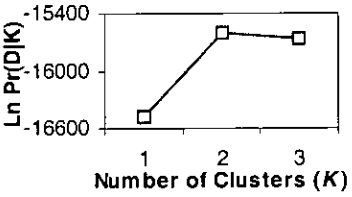
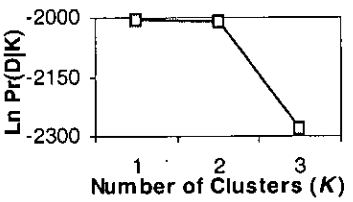
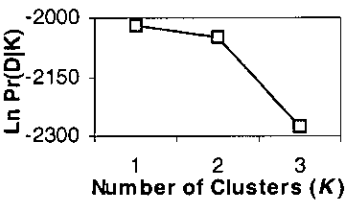
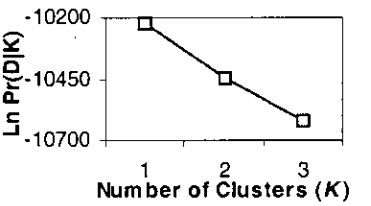
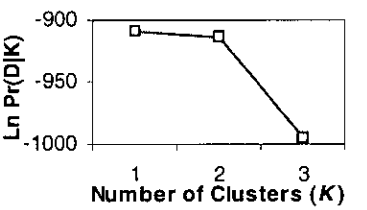
	K	Ln(PrD|K)	Plot of Ln probability of the data given each value of K
Site 1	1	-2991.06	
	2*	-2854.18	
	3	-2849.36	
Site 2	1	-9402.8	
	2*	-9067.4	
	3	-9242.1	
Site 3	1	-11730.1	
	2*	-11348.2	
	3	-11473.3	
Site 4	1	-16471.7	
	2*	-15600.7	
	3	-15663.0	
Site 5	1*	-2006.4	
	2	-2010.9	
	3	-2281.2	

Table 3.5. continued.

Site 6	1*	-2019.5	
	2	-2051.2	
	3	-2276.2	
Site 7	1*	-10224.8	
	2	-10443.3	
	3	-10620.3	
Site 8	1*	-909.8	
	2	-914.1	
	3	-995.1	

In addition to the spatial overlap of anadromous and freshwater sticklebacks in the river, we observed substantial temporal overlap in breeding seasons (Figure 3.6). In Sites 2-4, genetically freshwater females were in reproductive condition earlier than genetically anadromous females, but the breeding season of the two morphotypes overlapped for at least three months. We believe the core anadromous migration occurred between April and May because we did not observe any sticklebacks in the rock pools until May. This would also explain the low proportion of gravid anadromous females in Sites 2-4 in April. During the peak breeding period (May-June), anadromous females in Sites 2-4 greatly outnumbered freshwater females (114 anadromous females, 22 freshwater females, data not shown). The proportion of gravid hybrid females is similar to

freshwater females in April but noticeably declines from April through to June. In July, we observed a large proportion of gravid females in Sites 1 and 7, but relatively few in sympatric Sites 2-4.

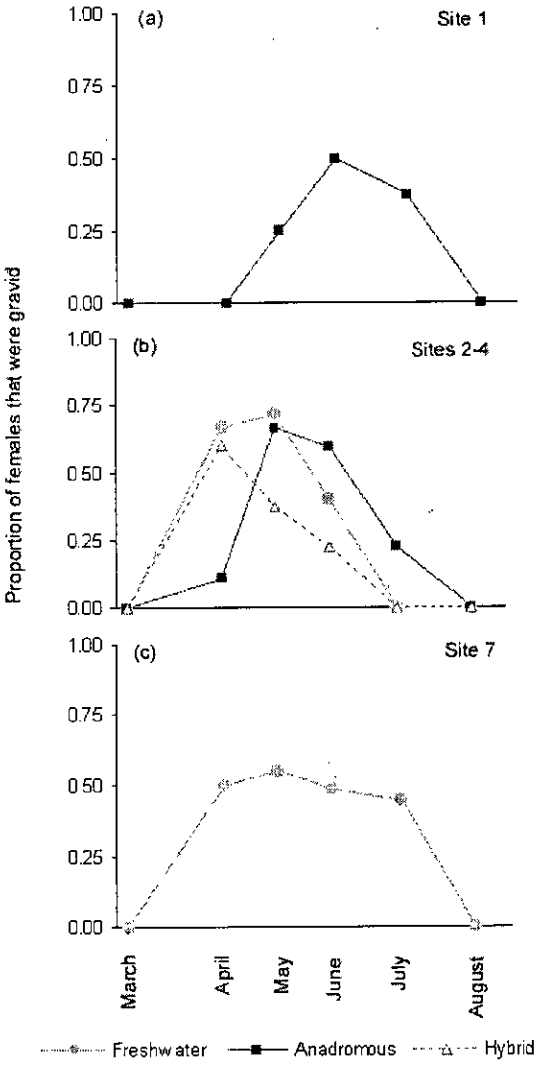


Figure 3.6. Temporal distribution of gravid females in (a) Site 1, (b) Sites 2-4 and (c) Site 7. Samples sizes for plots: Site 1 = 34, Sites 2-4 freshwater = 85, hybrid = 34, anadromous = 163, Site 7 = 103.

None of the 90% confidence interval estimates around *q* incorporated both 0 and 1, indicating our discriminatory power was strong enough to distinguish between anadromous and freshwater individuals. Further, we were able to identify many hybrid individuals whose confidence interval estimates around *q* did not encompass the anadromous or freshwater ends of the scale. The majority of hybrid individuals were sampled from Sites 2-4 where they comprise 33% of the juveniles sampled in July and August. Low frequencies of hybrids were sampled from upstream sites 5 – 8 where they comprise between four and six percent of the population sampled. The sampling of hybrids within sympatric sites indicates that premating isolation is not complete.

Reproductive Isolation

In Sites 2, 3 and 4, we found that the frequencies of genotypes of juveniles collected in July and August did not represent those expected from a randomly mating population. Over all loci, there was a significant deficit of heterozygotes compared to frequencies expected under Hardy Weinberg Equilibrium (Table 3.6). In contrast, the frequency of genotypes over all loci in samples of juveniles from upstream sites 7 and 8, did not deviate significantly from those expected under Hardy Weinberg Equilibrium. We observed consistent heterozygote deficits at the loci STN 152 and STN 26 in the sympatric sites, but no significant deficit in the remaining 8 loci. The strong heterozygote deficits at loci STN 152 and STN 26 appear to be causing the observed overall deficit of heterozygotes. Three possible scenarios can explain the observed pattern of heterozygote deficits at some loci but not others. Firstly, weak assortative mating may be occurring. Secondly, in a randomly mating population selection may be acting against eggs, fry or juveniles and this may be acting on particular gene

combinations (such as those present in individuals heterozygote at the loci STN152 and STN 26). Thirdly, null alleles may be present in the anadromous population at the loci STN152 and STN 26. The null alleles may not exist in the freshwater population since a heterozygote deficit was not observed at these loci in samples from Sites 7 and 8. At the locus STN26, F_{IS} values were slightly elevated in some samples from upstream sites 7 and 8 (Appendix Table 4), but F_{IS} values were consistently higher in Sites 2-4. However, a different pattern was not observed at the locus STN152, where F_{IS} values were found to be consistently low in samples from Site 7 and 8 but elevated in samples from sympatric Sites 2-4 and also in Site 1 (Appendix Table 4). This pattern would be consistent with the presence of null alleles in the anadromous genepool.

In juveniles sampled in July and August from Sites 2-4, individuals with anadromous mitochondrial haplotypes were more common than freshwater mitochondrial haplotypes (Table 3.7). We investigated cytonuclear disequilibrium and detected significant overall departures from random genotypic associations ($p<0.001$). The freshwater mitochondrial haplotype showed strong association with the freshwater nuclear genotype, and, similarly the anadromous mitochondrial haplotype showed strong association with the anadromous nuclear genotype (Table 3.7). This provides further evidence for either assortative mating or post-mating selection against fry. In contrast, we did not detect significant disequilibria with hybrid genotypes suggesting that the anadromous mitochondrial haplotype is sampled in hybrids as frequently as the freshwater mitochondrial haplotype. These data suggest that hybridisation between anadromous and freshwater sticklebacks is not biased in either direction.

Table 3.6. Results of HWE tests for heterozygote deficit in juveniles collected in July and August. NS not significant, * p<0.05, ** <0.01, ***p<0.001. Shaded cells are significant after sequential Bonferroni correction for multiple tests (Sokal and Rohlf 1995). Sites 1, 5 and 6 were not tested because juvenile samples collected in July and August were not genotyped (see Table 3.2).

Locus	Site 2	Site 3	Site 4	Site 7	Site 8
ATP1a2	NS	NS	NS	NS	NS
MyoHC3	NS	NS	NS	NS	NS
bAR2	NS	NS	NS	NS	NS
STN130	NS	NS	NS	NS	NS
STN152	***	***	***	NS	*
STN208	NS	NS	NS	*	NS
STN26	***	***	***	NS	NS
STN9	NS	NS	NS	NS	NS
STN94	*	NS	***	**	NS
STN96	*	NS	NS	NS	NS
Overall	***	***	***	*	NS

Table 3.7. Individual cytonuclear genotypic disequilibria (D* normalised disequilibria) for each of the three nuclear genetic ancestry groups at sites 2-4 combined. In this analysis, a negative D* represents an excess of anadromous mitochondrial haplotypes associated with a particular genotype, and a positive value an excess of freshwater mitochondrial haplotypes associated with a particular genotype.

	Mitochondrial Haplotype		D*	p value
Nuclear Genetic Ancestry (q)	Anadromous	Freshwater	Sites 2,3,4 Pooled	
Anadromous	114	5	-0.8380	*<0.0001*
Hybrid	54	24	0.0902	*0.4954*
Freshwater	9	33	0.7107	*<0.0001*

DISCUSSION

Anadromous and freshwater sticklebacks in the River Tyne differed significantly in morphology. Anadromous fish had more lateral plates, were larger, more robustly shaped and females possessed longer spines than freshwater sticklebacks. These differences are consistent with other studies (Hagen 1967, McPhail 1994, Ziuganov 1998, Scott 2000, McKinnon 2000, 2004) and provide further support for parallel evolution of morphological differences among freshwater sticklebacks. The significant genetic differences between anadromous and freshwater sticklebacks also mirrors other studies (Hagen 1967, McKinnon 2004) but contrasts with the findings of Higuchi *et al.* (1996) in their study of coexisting anadromous and freshwater fish in Lake Harutori. They found no evidence of a heterozygote deficit in breeding adults and concluded that these forms make up a single breeding population despite noticeable divergence in size. Genetic differences between anadromous and freshwater sticklebacks in the River Tyne indicate that some degree of reproductive isolation exists. Here we have presented evidence suggesting that either premating isolation assortative mating) or early hybrid mortality (postmating isolation) contribute, at least in part, to reproductive isolation between anadromous and freshwater sticklebacks in the River Tyne. This is the first time reproductive isolation between sympatric anadromous and freshwater sticklebacks in the wild has been reported.

During the breeding season, anadromous and freshwater sticklebacks overlapped spatially for at least three kilometres in the lower reaches of the river. Hagen reported that stream gradient limits the upstream migration of anadromous fish in the Little Campbell River. We found a similar effect here in the sharp transition in genetic

ancestry and morphology observed between sites 4 and 5 corresponding to the lowest weir on the river. Below the weir anadromous fish were present at high density, but they were completely absent above it. This suggests that the weir prevents upstream migration by anadromous sticklebacks despite the presence of a fish ladder. The fragmenting effect of weirs on gene flow has been reported in other river systems (e.g. Meldgaard *et al.* 2003). It is likely that this weir is contributing to premating isolation, acting as a barrier to hybridisation between anadromous and freshwater sticklebacks by limiting overlap in spatial distribution. It is likely that hybrids sampled at low frequency in sites above the weir (Sites 5-8) are individuals of freshwater ancestry with rare introgressed anadromous alleles. Within sites of overlap (Sites 2-4), we observed no differences in habitat preferences on a large scale, since we usually caught both morphotypes in any given trap. However, this does not preclude the possibility of microhabitat preferences occurring on a very fine scale and we are therefore unable to rule out the possibility that differences in microhabitat preference, particularly in the location of breeding sites, may also contribute to premating isolation in this system.

A substantial temporal overlap in the breeding season of anadromous and freshwater sticklebacks occurs in the months of April through to June. This differs considerably to the one-month overlap observed in the Little Campbell River, where the anadromous breeding season occurs later than that in the River Tyne (River Tyne: May – July, Little Campbell River: June – September). In the River Tyne sympatric sites 2-4, a slight temporal difference in breeding season was observed, with the breeding season of freshwater fish starting and finishing earlier than that of anadromous fish. From our mark-recapture data (Jones *et al.* in prep), we know that the resident freshwater

sticklebacks in this river have a one-year lifespan and the decline in gravid females between June and August follows closely the decline in population size due to adult mortality. In contrast, we do not know if anadromous fish survive for more than one year, therefore we are unable to rule out the possibility that the decline in adult anadromous sticklebacks is caused by migration back out to sea, rather than senescence. Even the small temporal difference in breeding season we observed may contribute to premating isolation between anadromous and freshwater sticklebacks by limiting the potential for hybridisation in the early and later months of the breeding season. However, in Sites 2-4 the bulk of the fish breed in both temporal and spatial sympatry.

Individuals of both intermediate genetic ancestry and morphology are found in sympatric sites supporting the hypothesis that hybridisation is occurring and is relatively common. Genetic hybrids represent 33% of juveniles sampled from sympatric Sites 2-4 in July and August. The observed hybrid frequency is lower than Hagen's (1967) estimate of hybrid frequency in sympatric sites in the Little Campbell River (46%). Differences in criteria used to define hybrids in these two studies means comparisons must be interpreted cautiously, however, it is possible that the difference in hybrid frequency is due to stronger reproductive isolation between anadromous and freshwater sticklebacks in the River Tyne. We argue that mating is not random because we detected both a significant heterozygote deficit and significant cytonuclear disequilibrium in juveniles sampled in July and August, however, selection against hybrid fry or eggs is an alternative explanation for these results. Although the heterozygote deficit was not strong across all loci, when combined with the observation

of cytonuclear disequilibrium these data provide good evidence for the existence of reproductive isolation between anadromous and freshwater sticklebacks. This finding conflicts directly with our previous study showing lack of assortative mating between fish from this river conducted in out-door ponds (Chapter 2).

Many factors differed between our previous assortative mating experiment performed in semi-natural conditions (Chapter 2), and the present study of assortative mating in the wild (Chapter 3). Ecological conditions such as flow, water depth, substrate, and microhabitat differed between the two studies and ecology-dependent mating may explain the possible existence of assortative mating in the wild but not in the ponds. In addition, the ratio of anadromous:freshwater females in the pond experiment was 1:1, but in the field was observed to be anadromous-biased (approximately 5 :1) during the peak breeding season. The density of wild fish in the River Tyne is also unknown and is another factor that may affect the strength of assortative mating and thus explain the potential conflict of the current results with the previous study.

It is difficult to ascertain the relative contribution of temporal differences in breeding season, possible differences in microhabitat use, and assortative mate preference, to the observed reproductive isolation. There is ample evidence of assortative mate choice occurring between anadromous and freshwater morphs sampled from allopatric populations (Hay and McPhail 1975, Scott 2001, McKinnon 2004) and this might be associated with differences in male courtship behaviour (Hay and McPhail 1975). However, Hay and McPhail (2000) found no significant association between the frequency of male courtship behaviours and male morphotype using individuals

collected from within two hybrid zones. Because of the large temporal overlap and lack of large scale microhabitat segregation between the two morphs, we argue that if the observed heterozygote deficit in juveniles is a result of premating isolation between anadromous and freshwater sticklebacks, then it is most likely explained by assortative mating.

Early hybrid fry mortality is an alternative explanation for the observed heterozygote deficit and presence of cytonuclear disequilibrium in juveniles. Our sample of juveniles consisted of individuals big enough to catch in our traps (mean standard length of juveniles sampled in Jul – Aug: 35.53mm ± 3.94 SD) and may already have been subject to selective mortality. However, laboratory crosses of anadromous and freshwater plate morphs have found no evidence of genetic incompatibilities and F1 hybrids and backcrosses are viable (Hagen 1967, McPhail 1994). In our previous study of assortative mating in semi-natural conditions (Chapter 2), there was no evidence of a deficit of fry (<10mm) from matings between anadromous and freshwater morphotypes compared to that expected under random mating. This observation provides further support for the lack of genetic incompatibilities in hybrid offspring, or at least, that genetic incompatibilities during development from egg to fry <10mm are not strong. This does not rule out the possibility of hybrid fry mortality being ecologically dependent, and this has yet to be studied in the wild.

Our tests for cytonuclear disequilibrium in hybrids revealed no directional bias in hybridisation events. However, in terms of absolute number, we observed many more hybrid juveniles with anadromous mitochondrial haplotypes than freshwater

mitochondrial haplotypes (Table 3.7). This is most likely due to the greater number of anadromous adults in these sites (Figures 3.4a and b) but might also be a result of higher fecundity of anadromous females (Hagen 1967, Chapter 2).

When comparing the distribution of *q* in adults collected from sites 2-4 to that of juveniles collected from the same sites we observed fewer adults of intermediate genetic ancestry than juveniles. This may be a result of yearly differences in the number of hybrids produced, or alternatively may be caused by postmating isolation (selection against hybrids). No direct evidence currently exists for postmating isolation in an anadromous and freshwater stickleback hybrid zone and this should be investigated further by studying the fitness of individuals from the same cohort throughout their life.

Several studies have identified the possibility of genetic differences between anadromous fish breeding in saline environments and anadromous fish breeding in freshwater (e.g. Saimoto 1993) or early and late run anadromous sticklebacks (Hagen 1967). This is unlikely to be the case in the River Tyne because genetic cluster analysis of all samples from all sites strongly indicate two rather than three genetic clusters being most likely. Nevertheless, we identified two distinct genetic clusters in our sample of individuals from the rock pools at Site 1 when tested on their own. This is unlikely to represent two groups of genetically distinct anadromous fish since it was not upheld in our analysis of all samples. Furthermore, the small sample size and high allelic diversity of fish from Site 1 precludes any specific conclusions. It is possible that lack of natal homing or high rates of straying result in genetic mixing between

anadromous populations (but see Saimoto 1993). Little is known about natal homing behaviour in anadromous sticklebacks and this warrants further attention.

This study offers three advantages over other studies of reproductive isolation in sticklebacks. Firstly, we studied reproductive isolation in a natural population, secondly, we were able to use genetic criteria to identify anadromous, freshwater and hybrid/introgressed fish, and thirdly, using genetics we investigated matings between sympatric anadromous and freshwater sticklebacks. We conclude that anadromous and freshwater sticklebacks in the River Tyne are not completely reproductively isolated and that hybridisation occurs often. However, we argue that either premating isolation or early selection against hybrid fry plays a role in maintaining divergence between the morphs. In the River Tyne, premating isolation may result from differences in the timing of the anadromous and freshwater stickleback breeding season, as well as from differences in microhabitat preference or ecology-dependent assortative mating. This is the first evidence of reproductive isolation between anadromous and freshwater sticklebacks in the wild.

CHAPTER FOUR

POSTMATING ISOLATION IN A STICKLEBACK HYBRID ZONE

ABSTRACT

Studies of selection acting on hybrids can provide a powerful approach for understanding the immediate selective pressures influencing the evolution and maintenance of morphological differences between anadromous and freshwater sticklebacks. In this paper, we provide the first evidence for postmating isolation in an anadromous-freshwater stickleback hybrid zone from a field-based study. We present clines in genes and quantitative traits across a stickleback hybrid zone located in the River Tyne, Scotland. The position of the centre of the mitochondrial cline was significantly further upstream than the centre of clines at nuclear loci, consistent with historical movement of the hybrid zone or different locations of environmentally-mediated selection gradients. We also observed a significantly narrower cline in standard length, compared to lateral plate number (LP) and shape, suggestive of greater selective pressure acting on the former trait. We calculated that the effective selection pressure (s*) required to maintain a single cline of the observed width to be between 0.17 and 0.58. This probably represents a maximum estimate since the estimate of dispersal on which it is based is likely to be inflated by the occurrence of assortative mating and selection against hybrid fry. Using quadratic regression we explore associations between indirect measures of fitness and genetic ancestry (q) and LP. We found a significant association between intermediate q and reduced probability of overwinter survival. Intermediate q was not associated with smaller juvenile standard length or reduced probability of female gravidity. In females, we also found intermediate LP was associated with smaller standard length, reduced overwinter survival and reduced probability of being gravid. Since these associations were not observed in males, our results are suggestive of sex-biased selective pressures operating in this system. Further, the

association between female fitness and LP but not q, suggests that genes controlling LP are closely linked to fitness genes of major effect. The manifestation of this in females and not males could be explained by pleiotropic interactions involving the X chromosome, but might also stem from sex-biased interactions with the environment.

INTRODUCTION

The three-spine stickleback (*Gasterosteus aculeatus)* is an excellent model organism for studies of evolution and speciation because of its recent adaptive radiation throughout the Northern hemisphere (McKinnon and Rundle 2002). Genetic evidence suggests that freshwater populations of sticklebacks have arisen from the repeated and independent invasion of freshwater habitats by marine sticklebacks. The repeated pattern of morphological divergence is suggestive of parallel adaptation to alternative environments. Throughout their distribution, morphological and genetic differences between anadromous and freshwater sticklebacks are being maintained despite the occurrence of hybridisation in sites of sympatry. These sympatric populations of anadromous and freshwater sticklebacks provide opportunities to study the roles of premating and postmating isolation in the evolution of reproductive isolation.

The extent of reproductive isolation between anadromous and freshwater sticklebacks appears to vary. Ziuganov (1995) reports complete reproductive isolation in plate morphs from Lake Azabachije, Russia, whilst Hagen (1967)

estimated hybrids represent 46% of individuals in the Little Campbell River (Canada) hybrid zone, Canada. Within the River Tyne (Scotland) hybrid zone, we estimated hybrids to represent approximately 39% of juveniles (Chapter 3). A number of studies have shown that anadromous and freshwater morphs hybridise readily, in semi-natural (Chapter 2) and under laboratory conditions (Hagen 1967, McPhail 1994), and morphs collected from sympatric sites show only weak assortative mating behaviour (Chapter 2). However, few studies have investigated premating or postmating isolation in wild populations (Hagen 1967, Chapter 3). A recent study of a hybrid zone found that premating isolation is likely to play some role in divergence between the morphs but reproductive isolation is not complete (Chapter 3). Individuals of intermediate morphotype have been reported in many populations (Hagen 1967, McPhail 1994), and in a previous study (Chapter 3) we found individuals of intermediate genetic ancestry. F1 hybrids of anadromous and freshwater morphs from the Little Campbell River showed no evidence of reduced fitness or inviability under laboratory conditions (Hagen 1967), and backcrosses are fertile (McPhail 1994). To date, very little is known about factors affecting postzygotic isolation or the nature of selection (if any) acting on hybrid sticklebacks within a hybrid zone. Using a field-based approach, we set out to investigate selection acting in a natural anadromous and freshwater stickleback hybrid zone.

Hybridisation is often believed to ultimately result either in the fusion of two races through introgression or alternatively, speciation through reproductive isolation. Yet there are many hybrid zones that appear to be stable. This stability can be explained by a variety of models. For example, the hybrid zone may be maintained by a dynamic equilibrium between migration of parental phenotypes into the zone and selection against hybrids, or alternatively, by increased hybrid fitness within the

zone compared to parental phenotypes but reduced fitness of hybrids once they move outside the zone (Moore 1977). In hybrid zones maintained by a balance between selection and migration, the coincidence (similarity of position) of clines in different traits is a common feature and may result from a variety of different processes (Butlin *et al.* 1991). In contrast, smooth coincident clines would not necessarily be expected under the hybrid superiority hypothesis, because the fitness of different hybrid phenotypes would depend on the location of different environmental gradients (Hewitt 1988). The hybrid superiority hypothesis may be particularly appropriate with regard to contact between anadromous and freshwater sticklebacks (Moore 1977). In 1967, Hagen looked for evidence of hybrid inferiority in an anadromous freshwater stickleback hybrid zone in the Little Campbell River, Canada. He found no evidence of reduced overwinter survival within the hybrid zone, and proposed that the sharp clines in morphology between sticklebacks must be maintained by selection against introgressed phenotypes outside the hybrid zone.

Hybrid zones have many characteristics that can be studied to infer processes contributing to the maintenance of species divergence. Stable hybrid zones result in distinct clines in genetic and phenotypic traits. Within a hybrid zone a cline is maintained by a balance between the influx of individuals from outside the zone and the selective removal of recombinant genotypes through exogenous selection (due to environmental factors), and/or by endogenous selection (selection due to genetic incompatibilities e.g. against heterozygotes) (Szymura and Barton 1986). This can be detected as associations between traits or loci (linkage disequilibria, D) and occasionally, as deviations from Hardy Weinberg proportions within loci. A heterozygote deficit (F_{IS}) in a population, results from non-random mating or

selection against hybrids, and changes in F_{IS} over time within a cohort may provide evidence of selection for or against hybrids. In the absence of selection, a heterozygote deficit collapses completely after a single generation of random mating. In contrast, linkage disequilibrium is broken down by recombination and, therefore, decays by a factor of 1-r (recombination rate) per generation under random mating. Linkage disequilibrium, therefore, reflects the extent to which alleles remain together despite interbreeding and its greater persistence than heterozygote deficit makes it a useful tool in studies of hybrid zones. A common characteristic of hybrid zones maintained by dispersal-selection balance is the coincidence of cline centres for numerous traits. This is thought to stem from secondary contact between populations that diverged in geographic isolation and also as a result of disequilibria between genes forming a genetic barrier. In contrast, if the strength or type of selection acting on traits or loci differs across the hybrid zone, then the degree of introgression will also differ, resulting in clines of varying width or shape. Dissimilarity in clines can be investigated by comparing parameter estimates for cline position and shape or, alternatively, by examining differences in the magnitude and pattern of change of D across the zone. This is possible because the magnitude of D is a function of the slope of the cline, with maximum values being found at the steepest part of the cline (i.e. the centre). As a result, wider clines show weaker disequilibria (Szymura and Barton, 1986).

Another way to investigate selection in hybrid zones is to consider fitness as a quantitative trait and examine the association between the change in relative fitness and change in a trait (e.g. Lande and Arnold 1983). In a linear relationship, the fitness gradient represents the slope (β_1) of a regression of the fitness measure (ω) against the trait (z):

$$\omega = \text{intercept} + \beta_1 z + \text{error}.$$

If a non-linear relationship exists, the nature of the fitness gradient can be examined by using quadratic regression where the fitness of an individual is regressed against the trait value (z) and it's squared term (z^2):

$$\omega = \text{intercept} + \beta_1 z + \beta_2 z^2 + \text{error}.$$

The significance and magnitude of the estimated quadratic coefficient β_2 describes the nature of the fitness selection gradient (Lande and Arnold 1983). A non-significant quadratic term is indicative of a simple linear relationship between the trait being investigated and fitness ($\omega = \text{intercept} + \beta_1 z$), with individuals of intermediate trait value showing no sign of either inferior or superior fitness compared to those with extreme trait scores. A significant positive β_2 coefficient suggests a concave fitness relationship (U – shape) and disruptive selection, whilst a significant negative β_2 coefficient suggests a convex fitness relationship (∩ - shape) and hybrid superiority. Differentiation of the predicted model provides a simple way of calculating the trait value conferring minimal or maximal fitness. Although requiring large samples sizes, quadratic regression provides a useful method of assessing hybrid fitness relative to individuals from parental source populations, and has been used in many studies of fitness (see Lande and Arnold 1983, Kingsolver 2001, Endler 1995).

In the present study, we investigated the maintenance of morphological and genetic divergence between anadromous and freshwater sticklebacks. We had several

aims: 1) To look for evidence of dissimilarity in cline shape and position for both genetic and quantitative traits. 2) To calculate the strength of effective selection, s*, that is required to maintain clines of the observed width. 3) Investigate changes in D across the zone using D estimates calculated for both genotypic and quantitative traits following the methods of Szymura and Barton (1986) and Nurnberger *et al.* (1995) and ask whether differences in the estimates of D made using alternative traits are evidence of variation in the intensity or spatial distribution of selection pressures. 4) Investigate changes in the strength of heterozygote deficit (F_{IS}) in a cohort over time. 5) Investigate associations between traits and individual fitness at three different stages of ontogeny.

The linear nature of river systems lend themselves well to one-dimensional cline analysis since migration can only occur in an upstream or downstream direction. However, the migratory behaviour of anadromous sticklebacks adds complexity due to changes in population composition throughout the year. Anadromous fish spawned in freshwater and are thought to undertake marine migration at approximately 2-3 months of age in late summer (Guderley 1994). They return to estuarine and freshwater habitats the following spring and breed throughout the summer. Although resident freshwater sticklebacks typically live for one year in this system (Jones *et al.* in prep.), it is possible that anadromous fish spend multiple seasons at sea or may embark on a second marine migration and participate in more than one breeding season (Fitzgerald *et al.* 1994). Little is known about site philopatry of anadromous sticklebacks, however, a single study reports that they return to their natal site and that site-specific homing occurs at a similar rate to those reported in trout or salmon (Saimoto 1989). Nothing is known about the migratory behaviour of hybrids.

In a previous paper (Chapter 2), we described the distribution of lateral plate number (LP) and genetic ancestry (q) in each of the sites across the hybrid zone. LP has often been used in the past as an indicator of an individual's genetic origin due to the ease of quantification (e.g. Ziuganov 1995 , Scott 2004, Hay and McPhail 1975). However, morphological traits within a hybrid zone may not provide an accurate reflection of an individual's genetic ancestry, since hybridisation produces recombinant genotypes and phenotypes, and neutral traits or genes may introgress faster than those exposed to strong selective influences. In this paper, therefore, we explore the correlation between LP and q within the hybrid zone and investigate the shape of fitness gradients associated with these traits using quadratic regression. Divergence in q and LP may be maintained solely by selection acting on individuals of intermediate trait value once they move outside the hybrid zone and there is therefore no reason to form an *a priori* prediction about hybrid fitness (inferiority or superiority) within the hybrid zone.

Rapid divergence in LP between anadromous and freshwater-resident sticklebacks since post-glacial colonisation is likely to be a result of strong divergent selection acting on the genes controlling plate morphology. A recent study by Bell *et al.* (2004) has shown that LP adaptation can happen on an extremely rapid timescale (e.g. 12 years) suggesting that selection pressures on this trait are intense. There is some evidence that predation pressure may play a key role in selecting LP, but the exact nature of this selection is unclear. Lateral plates act as armour by protecting the body from tissue damage by toothed predators that may be incurred during predator handling and manipulation. Lateral plates may be advantageous once caught, due to longer manipulation time and higher probability of escape after

capture (Reimchen 2000). The anterior plates are thought to decrease probability of ingestion by predators by providing a structural support link between dorsal and pelvic spines (Reimchen 1983). This is likely to be important in populations exposed to gape limited predators (Hoogland *et al.* 1957). However, there is likely to be a metabolic cost to plate production, and laboratory studies have shown that posterior plates may be detrimental by conferring a slower burst-swimming response (Taylor and McPhail 1986). Reimchen (1992) suggests further that a lack of lateral plates may help prevent capture by avian predators, which may be more abundant in freshwater environments. LP is also correlated with a number of physical variables including, climate (latitude, but see Sargent *et al.* (1984) where latitudinal clines in LP of sister species *Gasterosteus wheatlandi* are opposite to that of *Gasterosteus aculeatus*), stream gradient and temperature (Bell 1984, Baumgartner and Bell 1984). In addition, in some populations dissolved calcium levels may be correlated with plate and spine reduction (Giles, 1983), but this does not seem to be the case in Icelandic populations (Eik Olandsdottir, Pers. Comm.). To date, there has been no study of the relative fitness of plate morphs in a stickleback hybrid zone, yet this approach is likely to provide useful insight to the divergence of LP between anadromous and freshwater morphs.

We investigated fitness at three different stages of ontogeny since selection pressures acting on an individual are likely to change throughout its lifetime. Firstly, viewing standard length as an indirect fitness measure in juveniles, we examined the association between standard length and LP and q. Several studies have demonstrated the utility of using standard length as a fitness measure in sticklebacks. Body size is correlated with energy income and potential fecundity (Wootton, 1994). Body size and lipid content at the start of winter is also correlated

with the probability of overwinter survival in trout and salmon (e.g. Parrish *et al.* 2004), although standard length may not always be an accurate predictor of body condition. If hybrid inferiority was present in the juvenile stage then we would expect intermediate LP and/or q to be associated with smaller standard length. Conversely, if hybrid vigour was present in the juvenile stage then we would expect intermediate LP and/or q to be associated with larger standard length. Secondly, we investigated the association between over-winter survival and LP and q and, finally, we examined if the probability of females becoming gravid was associated with LP and q.

Our investigation of postmating isolation in sticklebacks was conducted in an anadromous–freshwater stickleback hybrid zone in the River Tyne, East Lothian Scotland. Our study system was located in the lower reaches of the river where both resident freshwater and anadromous morphs breed sympatrically. The one-year life-span of resident freshwater sticklebacks in this system (Jones *et al.* in prep.) made it easy for us to follow the same cohort throughout ontogeny. Previous studies suggest that assortative mating under semi-natural conditions is not strong (Chapter 2) and that hybridisation occurs in the wild (Chapter 3). The River Tyne is therefore a useful system for studying the nature of selection acting in a stickleback hybrid zone and the extent of postzygotic isolation between this anadromous and freshwater species pair.

MATERIALS AND METHODS

FIELD WORK AND SAMPLE COLLECTION

All work carried out in the field was performed with the assistance of Dr Culum Brown. Sticklebacks were sampled from eight sites along the River Tyne, East Lothian, Scotland. In order to understand changes in population structure over time, we carried out sampling of the hybrid zone in September 2002 and then on a monthly basis throughout 2003. Specific details of the sampling are described in Chapter 3 (see Tables 3.1 and 3.2). Briefly, the sites were concentrated in the lower reaches of the river and ranged from rock pools at the mouth (site 1), sites where both anadromous and freshwater morphs breed sympatrically (sites 2-4), and sites consisting predominantly of freshwater morphs (sites 5-8). Fish were captured in standard minnow traps and were fin-clipped for genetic analysis, tagged using a site-specific visible elastomer tag, photographed, and morphological measurements taken before being released back into the river at the site of capture. Our sampling strategy meant that we were able to compare samples of individuals collected from the same cohort (cohort 1) before winter (September 2002) and after winter (April – July 2003). In addition, we were able to sample the progeny of these individuals (cohort 2) over the first six months of their life (July – December 2003). All samples collected prior to April 2003 were killed using a UK Home Office Schedule 1 method and preserved in ethanol. After April 2003, all fish were tagged and returned to the river, enabling us to identify previously sampled individuals. During the period of our fieldwork, over 3500 fish were tagged and photographs, morphological measurements and fin-clips were taken from approximately 2000 of these fish.

MORPHOLOGY

Morphological data collected in the field included lengths of the left pelvic, first, and second dorsal spines, and lateral plate count (LP) on the left side of the body. Other morphological data was obtained from digital analysis of photographs by recording the x and y coordinates of eleven landmarks (concentrating around the head region to avoid shape changes in gravid females affecting our analysis) and additionally measuring the length of 4 other traits including standard length. Partial warp analysis was used to extract shape variables from landmark data, and a discriminant function analysis summarised shape differences between anadromous and freshwater sticklebacks into a single variable (see Chapter 2 for details).

GENETICS

In the laboratory, we selectively genotyped random samples of fish collected from the field (Table 4.1, see Chapter 3 for more details). 1961 fish were sexed genetically and genotyped at seven microsatellite loci, 3 nuclear intron single nucleotide polymorphisms (SNP's) and one mitochondrial locus as described in Chapter 2. Individual genetic ancestry (q) was calculated using the program STRUCTURE (Falush *et al.* 2003) to cluster individuals based on their nuclear multilocus genotypes (see Chapter 2 for details). STRUCTURE analysis revealed the presence of two distinct genetic clusters within our entire dataset which correspond spatially and morphologically to anadromous and freshwater populations (Chapter 2). Based on an individual's genotype at 10 nuclear loci, and representing the probability of an individual having ancestry from an anadromous source population, individual genetic ancestry coefficients (q) extracted from the STRUCTURE analysis were used as a genetic hybrid index.

Table 4.1. Sample sizes of fish genotyped from each site each month. Age Class A = Adult, J = Juvenile. Distance represents location of distance of site from ocean in kilometres.

Cohort		COHORT 1							COHORT 2							
Year		2002	2003													Grand
Month		Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Site
Age class		J	A					A	J						J	Total
Site	Distance															
1	0.1	-	-	34	20	16	1	71	-	-	-	-	-	-	0	90
2	4.0	45	14	38	50	1	-	103	39	50	27	5	3	6	130	278
3	4.5	45	23	42	50	5	-	120	32	50	50	30	13	15	190	355
4	6.5	50	50	45	35	7	-	137	22	50	50	30	30	23	205	525
5	7.25	45	-	-	-	-	-	0	-	-	50	-	-	-	50	95
6	8.0	45	-	-	-	-	-	0	-	-	50	-	-	-	50	95
7	9.25	45	30	36	50	39	-	155	11	50	44	30	30	30	195	473
8	19.3	-	-	-	-	-	-	0	-	50	-	-	-	-	50	50
Total		275	117	195	205	68	1	586	104	250	271	95	76	74	870	1961

CLINE ANALYSIS

Using genotype data, we were able to construct clines in allele frequency across the hybrid zone for the three nuclear intron loci, the mitochondrial locus, and q. Clines for microsatellite loci were not fitted firstly, because alleles were not diagnostic between anadromous and freshwater fish and secondly, because applying a subjective assignment of non-diagnostic alleles to either 'anadromous' or 'freshwater' populations at loci with many alleles (e.g. 62 different alleles were detected at locus STN152, Appendix Table 2) would introduce substantial error into the cline analysis. Clines for each locus were fitted based on allele frequencies in juvenile samples (Cohort 2) collected in September 2003. No juveniles were caught in the rock pools at site 1 in 2003 (possibly because they had already migrated out to sea), so we used the genotype frequencies calculated from adults (Cohort 1) in our clines under the assumption that the allele frequencies in juveniles would not differ significantly to that of adults at this site. Because a sample of juveniles from

Site 8 was not collected in September 2003 (see Table 4.1), juveniles collected from Site 8 in August were used instead. We used the program Analyze (Barton and Baird, 1996) to fit maximum likelihood clines to each loci. Clines for single loci maintained by a balance between dispersal and selection often have a sigmoidal shape (Szymura and Barton, 1986). This is described by a tanh curve as a function of its width (w) and centre (c):

$$p = (1+ \tanh[2(x-c)/w])/2)$$

where x represents the distance from the centre of the cline (Szymura and Barton 1986). More complex stepped clines, where a steep change occurs in the centre of the cline, might be expected when several traits change simultaneously across a hybrid zone due to the associations between loci. However, due to the limited number of sampling sites across the hybrid zone we refrained from fitting more complex stepped cline models since this would involve estimating four or six parameters from a data set with only eight collection points. Because allele frequencies at the nuclear intron loci were not fixed at 0 and 1 at either end of the cline, we constrained the model by setting p_{min} and p_{max} to the observed allele frequencies at site 1 and 8 where they represent allele variation in anadromous and freshwater populations respectively.

$$p = p_{min}+(p_{max}-p_{min} (1+ \tanh[2(x-c)/w])/2)$$

Support limits for the genetic cline width and centre parameters were obtained by determining the maximum and minimum parameter estimates 2 units below the

maximum log likelihood, approaching an approximation of 95% confidence limits (Edwards, 1972).

Clines in quantitative traits were also constructed using a tanh curve, which is likely to be a reasonable approximation for clines in quantitative traits under polygenic control (Butlin *et al.* 1991). Quantitative trait clines were based on adults sampled from the breeding season in 2003 (April – July). We used adults for these clines to avoid complexity associated with potential differential expression of a trait in anadromous and freshwater juveniles during growth. The difference in the size of anadromous and freshwater fish is one of the major traits thought to contribute to reproductive isolation in anadromous and freshwater fish (McKinnon *et al.* 2004). We therefore analysed the cline of standard length in addition to clines in LP, and head shape, which were independent of body size. Also we initially set out to present clines in traits such as spine lengths, head depth, head length and eye diameter after adjusting for size, but found significant differences in allometry between sites for each trait. This was identified as significant differences between sites in the slope of the regression of log(trait) against log(standard length). It is likely that within a site in the middle of the hybrid zone individuals also differ in allometry making the correct adjustment for size very difficult. For this reason, we present clines in the size-unadjusted traits, but the effect of differences in allometry between sites should be kept in mind when interpreting cline parameters. Cline parameter estimates and confidence intervals for quantitative trait clines were calculated using the nls command in SPLUS 6.0 (Lucent Technologies Inc, 2001).

The coincidence of cline centres was tested by fitting a cline to one trait whilst constraining the cline centre to that of another trait. Concordance of cline width was

assessed in a similar manner by fitting a cline to one trait whilst constraining cline width to that of another trait. Change in likelihood (comparisons between genetic loci) and F-ratio tests (comparisons between quantitative traits) were used to determine if the constrained model was significantly worse than the fully parameterised model.

LINKAGE DISEQUILIBRIUM

Linkage disequilibrium results from non-random associations between alleles at different loci, and can be generated by the migration of individuals possessing co-adapted gene complexes into a hybrid zone. Other factors such as epistatic selection (selection for particular gene combinations) or assortative mating may also play a role. Across the hybrid zone we explored both linkage disequilibrium based on associations between alleles at different loci (D, or more specifically R) as well as linkage disequilibrium based on associations between traits (D*, or more specifically D_F). Linkage disequilibrium is dependent upon allele frequencies $D = p_{11} - p_1q_1$ (where p_{11} represents the frequency of the genotype p_1q_1 and p_1q_1 represent the frequencies of the alleles p_1 and q_1 in the population respectively) and this relationship constrains the value of D to being smaller in populations that are less polymorphic. This makes comparisons of D between populations varying in allele frequencies difficult. We therefore present maximum likelihood estimates of the standardised linkage disequilibrium (R, Szymura and Barton 1991) averaged over all pairs of loci. This value lies between −1 and +1, but does not entirely correct for effect of allele frequencies since the full range of R values from −1 to +1 can only be achieved in populations were p=q=0.5 (Lewontin 1988) . These estimates are based on the nuclear intron SNP and mitochondrial markers and were obtained using the program Analyse (Barton and Baird, 1996).

Associations (or covariances) between quantitative traits are also of interest because they reflect associations between underlying sets of quantitative trait loci, and thus linkage disequilibrium. The calculation of linkage disequilibrium from quantitative traits (D*, Nurnberger *et al.* 1995) is based on estimates of the covariance between traits and makes several fundamental assumptions: firstly, that the effects of genes influencing a trait are additive, secondly, that the covariance between traits is due entirely to covariance between the underlying allele states, thirdly, that differences in the loci underlying the trait are fixed in parental source populations, and fourthly that pleiotropy is absent.

Nurnberger *et al.* (1995) present a formal derivation of linkage disequilibrium based on quantitative traits:

$$D^* = (2\,\mathrm{cov}(z,z'))/(\Delta z \Delta z')$$

where disequilibrium between traits z and z' is proportional to twice the covariance in the traits, scaled by the differences in the traits across the hybrid zone (Δz and $\Delta z'$). This relationship assumes that the loci underlying the quantitative traits are in Hardy-Weinberg equilibrium, and therefore estimates of D* will be inflated upwards by non-random gametic assortment (e.g. F_{IS}). We do not correct for this in our analyses and refer to estimates of D* as D_F. In a given site, all estimates of D_F are likely to be biased to the same extent thus enabling comparisons between estimates.

Many quantitative traits change with growth and this covariance with size has the effect of inflating estimates of linkage disequilibrium. For these traits, it is therefore necessary to correct for size before performing analysis. However, as mentioned above, we found significant differences in allometry for all of the studied traits. We therefore only calculated linkage disequilibrium in traits not affected by growth (LP, shape, standard length, and q). Trait scores were mean-centred within each site to avoid differences in the magnitude of the trait across the hybrid zone affecting the estimated covariance.

DISPERSAL ESTIMATES AND STRENGTH OF EFFECTIVE SELECTION

In a cline maintained by a balance between selection and migration, it is possible to calculate the effective selection pressure (s^*) that would be required to maintain a cline of the observed width; $s^* = 8(\sigma/w)^2$ (Szymura and Barton 1986), providing an estimate of dispersal is available and assuming the balance between selection and migration has reached equilibrium. This selection coefficient is a measure of selective pressure against heterozygotes relative to other genotypes in the population (and is equivalent to $1 - \omega$, where ω is fitness of a particular genotype) and therefore ranges from 0 – 1. Estimates of dispersal per generation can be obtained from mark-recapture field data, or can be inferred in two ways from genetic data. Firstly, it can be estimated from the slope of the relationship between pair-wise population genetic distance (F_{ST}) and geographic distance, using populations outside the hybrid zone. Secondly, dispersal can be inferred from the relationship between cline width and linkage disequilibrium. Linkage disequilibrium will be greatest in the centre of the zone and will be proportional to the dispersal rate and the gradients of the clines (width is defined as 1/estimated gradient, Szymura and Barton 1986). Assuming that the observed linkage disequilibrium is primarily due to

migration rather than selection, it is possible to estimate the dispersal rate (σ) required to maintain the observed disequilibrium using the equation: $\sigma = w\sqrt{(R^*r/4)}$ (Szymura and Barton 1986) where w is the estimated cline width, R is the standardised linkage disequilibrium coefficient and r the recombination rate (0.5 assuming loci are unlinked). We are limited in the number of populations sampled outside the hybrid zone, and therefore calculate estimates of dispersal based on the maximum observed linkage disequilibrium (R) in the centre of the hybrid zone. We discuss this dispersal estimate in the context of a mark-recapture study carried out in the field (Jones *et al.* in prep), and use it to estimate the effective selection pressure acting against hybrids in this system.

ANALYSIS OF HETEROZYGOTE DEFICIT

The inference of selection acting against hybrids does not distinguish between selection occurring within the hybrid zone and selection acting on hybrids once they move out of the hybrid zone. We investigated hybrid fitness further by exploring changes in F_{IS} over time, and fitness gradients associated with particular traits. In a previous paper we reported a heterozygote deficit (F_{IS}) in juveniles collected from sites 2-4 (where hybridisation occurs; Chapter 3). Here, we investigate changes in F_{IS} over the course of 6 months in order to determine the fate of recombinant juveniles within the hybrid zone. We used the program FSTAT (v2.9.3.2, Goudet 2002) to investigate deviations from Hardy Weinberg equilibrium within samples, across all nuclear loci. If selection against hybrids occurs within the hybrid zone, we would expect to see an increase in heterozygote deficit in a cohort over time. Alternatively, if hybrid vigour exists in the hybrid zone, we would expect to see a decrease in heterozygote deficit over time. F_{IS} was calculated for each monthly sample collected from sites within the hybrid zone (sites 2-4) and from a freshwater

site (site 7). We then performed two statistical tests to examine changes in F_{IS} within a cohort. Firstly, we investigated the change in F_{IS} over winter using cohort 1, by comparing F_{IS} estimates of juveniles from sites 2-4 in September 2002 (N=3) to that of adults in sites 2-4 in April – July 2003 (N=9). The significance of this test was determined by permutation, where the observed difference between the two groups was compared to the difference between two groups consisting of randomly assigned samples. The p-value for this test represents the proportion of randomised data sets giving larger differences than observed. Secondly, we investigated the change in F_{IS} in juveniles before winter using cohort 2. In this analysis, we pooled samples from site 2 in the months of October – December because of small sample sizes. We performed two separate linear regressions of F_{IS} estimates against the date of sample collection (one based on F_{IS} in samples from Sites 2-4 and, as a control, one based on samples from Site 7). The change in F_{IS} over time is likely to be complicated by the migration of anadromous juveniles out to sea and we interpret our results in the context of changes in the distribution of q over time.

ANALYSES OF FITNESS

Initially, we set out to investigate associations between several quantitative traits and indirect fitness measures. However, differences in allometry between sites in growth related traits made correcting for size before analysing fitness associations difficult (particularly within the hybrid zone where the growth allometry of an individual is likely to depend on its genetic ancestry). Instead we concentrated on two traits which are independent of growth: LP and q. We started by investigating the relationship between our genetic measure of hybrid status (q) and the quantitative trait LP in sites located within the hybrid zone (sites 2-4). Although we found the correlation between q and LP to be good (see results) it is apparent that

these two traits contain independent variation which warrants the inclusion of LP and q as independent explanatory variables in our fitness models. Our interpretations of the presence of hybrid inferiority/superiority are based on the results of quadratic regressions. In using a quadratic to describe a non-linear relationship we make particular assumptions about the shape of that relationship. Quadratic functions are symmetrical about the point of inflection, but we have no reason to assume that the fitness gradient we observe is symmetrical. A fitness surface might be bumpy, and in this regard, loess regression has advantages over quadratic regression because it makes no assumptions about the overall nature of the gradient. Further, the significance of a quadratic term does not automatically imply that the maximal or minimal fitness lies within the range of data being tested. Differentiation must be performed to determine this. The advantage of using a quadratic regression approach is that additional terms and interactions can be fitted and interpreted with ease.

Fitness Models

We used pooled individuals from sites 2-4 to explore the association between three different indirect measures of fitness and LP and q. We tested for the presence of non-linear associations of LP and q with the fitness traits by including the quadratic terms LP^2 and q^2 in each model.

I. Juvenile Standard Length:

We examined whether standard length of individuals was associated with LP and q in juveniles collected from July – December 2003 (Cohort 2). To remove the effects

of growth over time, we initially calculated individual growth residuals from a non-linear regression with an asymptotic curve

$$\text{Standard Length} = a - b * e^{(-c * \text{Day})}$$

where e represents the constant 2.718 (the natural log of 2.718 = 1), the parameter a defines the asymptote, and b and c influence the gradient. Residuals from this growth curve represent the size of an individual in a given month relative to the population growth curve. Individuals of large standard length in a given month will have positive residual score, whilst individuals of small standard length in a given month will have negative residual score. Although juvenile standard length was adjusted in this fashion, the resulting growth residuals nevertheless reflect a given juvenile's standard length and we continue to refer to this indirect measure of fitness as juvenile standard length throughout the rest of this chapter. We then investigated the amount of variation in growth residuals explained by a linear model (ANCOVA) with q, LP and their quadratic terms q^2, LP^2. In addition, sex and day were added as a factor and covariate respectively.

II. Overwinter survival:

We examined the distributions of both LP and q in juveniles and adults collected from cohort 1 (September 2002, April-July 2003). If selection against individuals of intermediate LP or q occurred over winter we would expect to find fewer intermediate adults than juveniles. Conversely, if individuals of intermediate trait value were more likely to survive winter than individuals of extreme trait value we would expect a higher proportion in the adult sample. In order to test our hypotheses, we treated juvenile/adult status as a binary response variable in a

generalised logistic linear model with Sex, q, q^2, LP and LP^2 as independent variables. Since the number of adults sampled was greater than the number of juveniles sampled and the y-intercept is dependent upon sampling regime, the y intercept of the fitted model cannot be interpreted directly. The slope estimates from this model, however, are unbiased and can be used to test hypotheses that a particular LP or q is associated with a higher probability of reaching adult status because they represent the relative survival rates of different individuals with various trait scores. By including the quadratic terms for q and LP, we were able to test for a significant non-linear relationship between these traits and the probability of overwinter survival.

III. Female Gravidity:

We examined whether reproductive status of females, who have already survived to adulthood, was associated with LP and q. We used a binary generalised linear model with gravid status as the response variable and date, standard length, LP, LP^2, q, and q^2 as independent variables. Adult females collected from April to July 2003 (Cohort 1) were used in this analysis.

Statistical analyses mentioned in the above three measures of fitness were carried out using the non-linear, least squares regression, linear model or generalised linear model functions in S-PLUS (v6.0, Lucent Technologies Incorporated, 2001). Models were minimised by sequential removal of higher order interactions that did not explain significant amounts of the variation using F-tests (juvenile standard length model) or Chi-tests (overwinter and female gravidity model).

RESULTS

CLINE ANALYSIS

The fitted clines for nuclear intron loci, q, and the mitochondrial locus Cytb are shown in Figure 4.1. There is a large degree of scatter around the fitted clines for the bAR2 and MyoHC loci. This, combined with the relatively small change in allele frequencies from sites 1 to 8, resulted in low confidence in the estimated cline widths and centres for these loci.

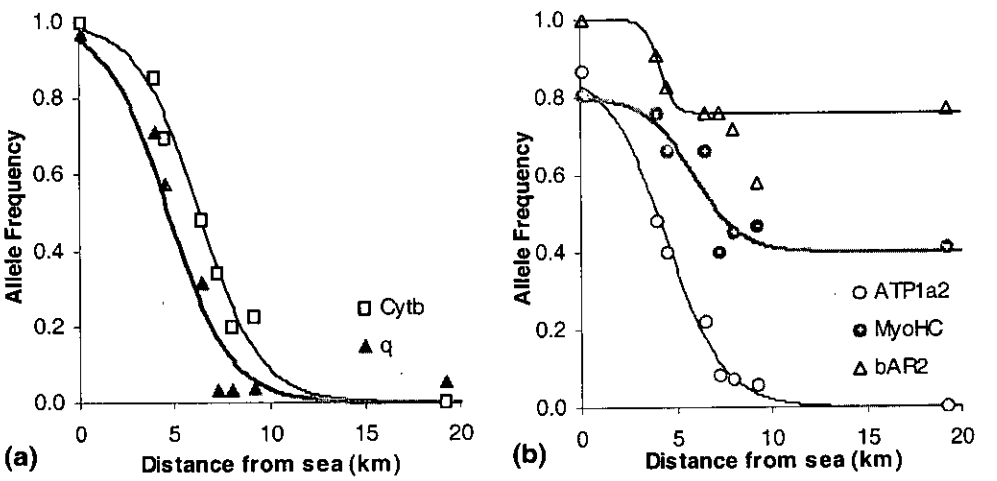


Figure 4.1. Clines in allele frequency across the hybrid zone from site 1 (Distance = 0 km) to site 8 (Distance = 19.3 km) for Cytb and q (a) and ATP1a2, MyoHC, and bAR2 (b). Fitted clines represent cline centre and width under the most likely sigmoidal model. Points represent mean allele frequency or q value at each site.

Quantitative trait clines are shown in Figure 4.2. We observed a gradual change in trait scores from sites 1-5, while the upstream sites (from site 5 – 8) showed similar mean trait scores. Although the limited number of sampling sites across the hybrid zone prevented fitting more complex clines to these data, visual inspection of the

clines reveals a sharp change in traits and allele frequencies between sites 4 and 5 that coincides with the presence of the lowest weir in the River Tyne. It is likely that this weir acts as a strong physical barrier to individual migration upstream.

We found a lack of coincidence in several pair-wise comparisons between cline centres (Figure 4.3). The centre of the Cytb cline (6.63km) was located significantly further upstream than both the centre of the cline in q (4.73km, F_1=49.583, p=0.0004), and the average centre position of the three nuclear intron loci (4.86, F_1=43.099, p=0.0005). The centre of the MyoHC intron cline (5.94km) also differed significantly from the average centre of the two other intron loci (4.32km, F_1=7.203, p=0.036) but did not differ significantly from the position of the centre of q (4.73km, F_1= 4.558, p=0.077). Analysis of position of clines in quantitative traits revealed the centre of the cline in standard length (6.55km) was located significantly further upstream than the average location of clines in shape and LP (5.35km, F_1=16.694, p=0.0065). We observed an upstream displacement in the position of the centre of the cline for the 2nd dorsal spine relative to the pelvic and 1st dorsal spine, however, this difference was only marginally significant (F_1=5.721, p=0.0539).

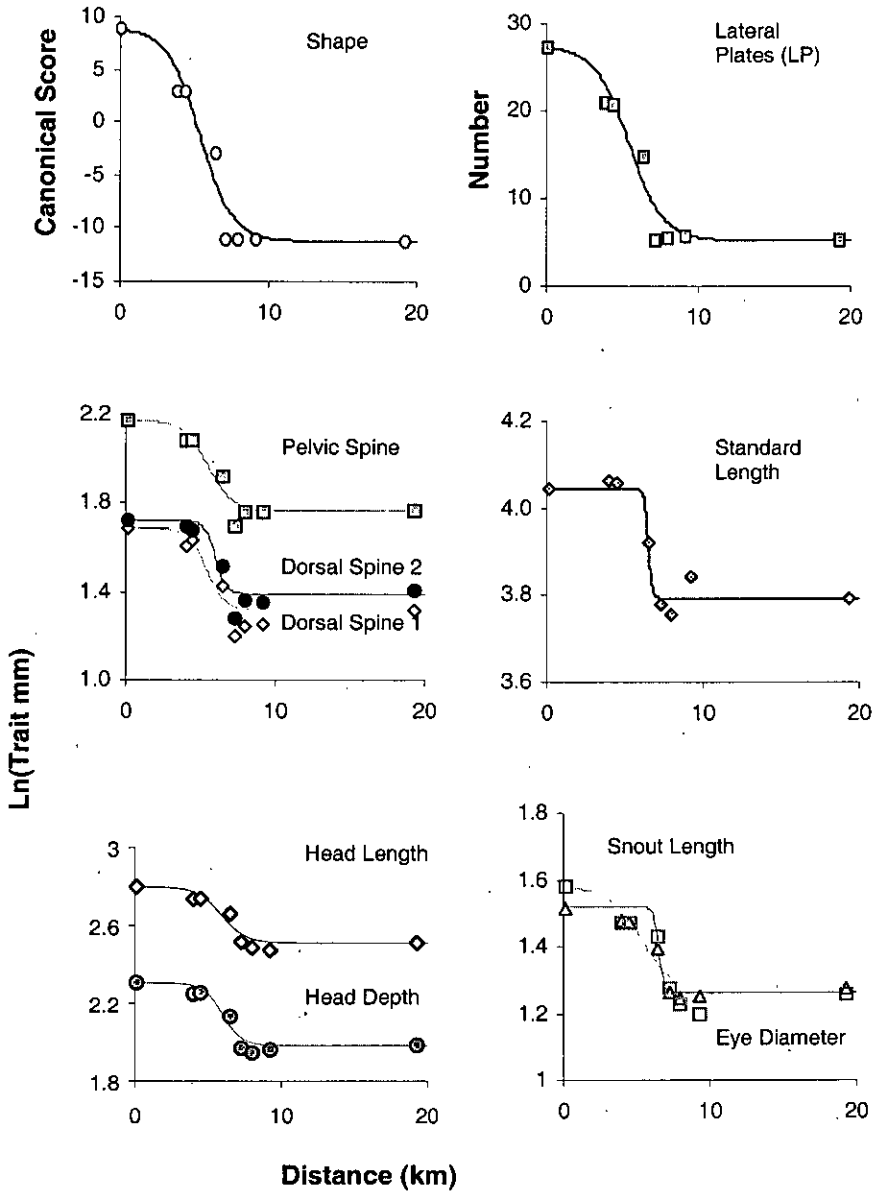


Figure 4.2. Fitted clines in quantitative traits across the hybrid zone from sites 1 (Distance = 0km) to Site 8 (Distance = 19.3km). Points represent mean trait score at each site.

We found no significant difference between the width of the bAR2 cline (1.42km) and the average width of clines of other genetic loci (5.87km, F_1=1.163, p=0.322). Constraining the minimum and maximum values of the bAR2 cline to the observed allele frequencies in sites 1 and 8 resulted in a noticeably poor fit for allele frequencies at site 7 (Figure 4.1). The resulting estimate of cline width for this locus (1.42km) is much narrower than that observed at other loci (5.87km) despite the absolute difference in allele frequency in sites 1 and 8 being small. If a cline is fitted to the same data allowing p_{min} and p_{max} to vary, the resulting width estimate is 4.87km. This is more similar to the estimates of cline width at the other loci but has large confidence intervals (0.300-14.24, 95% confidence intervals). The substantial overlap in 95% confidence intervals of clines width estimates for other genetic clines precluded the need to test for significant differences in cline width. The width of the cline in standard length (0.81km) was significantly narrower than the average width of the clines in plate number and shape (4.37km, F_1=10.773, p=0.0168).

We refrain from making comparisons between the position and width of genetic and quantitative trait clines because these clines were based on data collected from different individuals. At present, we lack genetic data on adults from sites 5 and 6 making estimates of genetic cline shape and width based on adults unreliable.

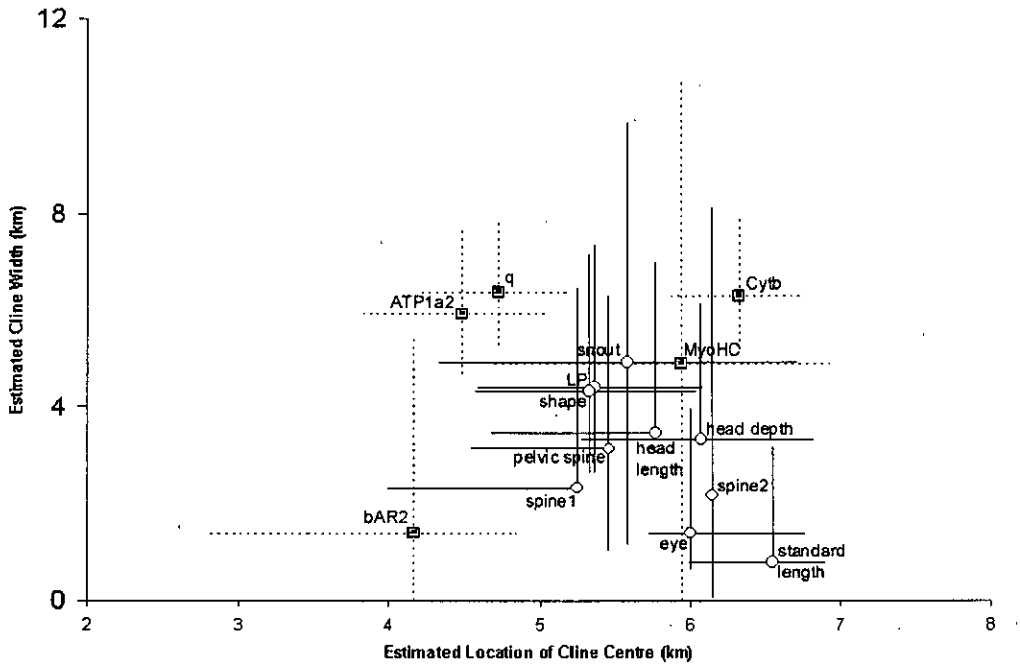


Figure 4.3. Estimated location of cline centre and cline width for genetic loci (solid squares), and quantitative traits (circles). Support limits represent two unit maximum likelihood limits (approximating 95% confidence intervals). Clines in quantitative traits were based on data collected from adults whilst genetic clines are based on data from juveniles.

LINKAGE DISEQUILIBRIUM

We detected significant standardised linkage disequilibrium (R) between loci in juveniles collected from sites 2 – 4 in September 2003 (Figure 4.4). The magnitude of linkage disequilibrium we observed is relatively high (maximum R value was in Site 3 = 0.313, 95% confidence intervals: 0.175-0.366) compared to estimates in other hybrid zones (e.g. R=0.129 (0.119-0.139) in *Bombina*, Szymura and Barton 1986). Examination of the distribution of q within these sites confirms that the high linkage disequilibrium observed was a result of individuals with extreme ancestry

scores. In this respect, the hybrid zone between anadromous and freshwater sticklebacks is highly bimodal. Estimates of linkage disequilibrium between quantitative traits (D_F) were also elevated in sites 2 – 4 (Figure 4.5), with highest estimates in females consistently found in site 4. In contrast, the greatest estimates of linkage disequilibrium between quantitative traits in males varied between sites 2 and 4. In a manner similar to the genetic estimates of linkage disequilibrium, the maximum values of our estimates of D_F within the hybrid zone are also high compared to those estimated in *Bombina* hybrid zone (Kruuk 1997, Sands 2005).

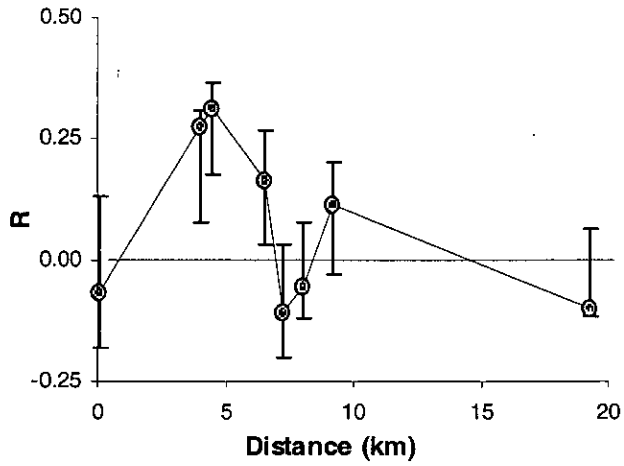


Figure 4.4. Standardised linkage disequilibrium across the hybrid zone from site 1 (distance = 0km) to Site 8 (distance = 19.3km). Two unit maximum likelihood support limits are approximately equivalent to 95% confidence intervals.

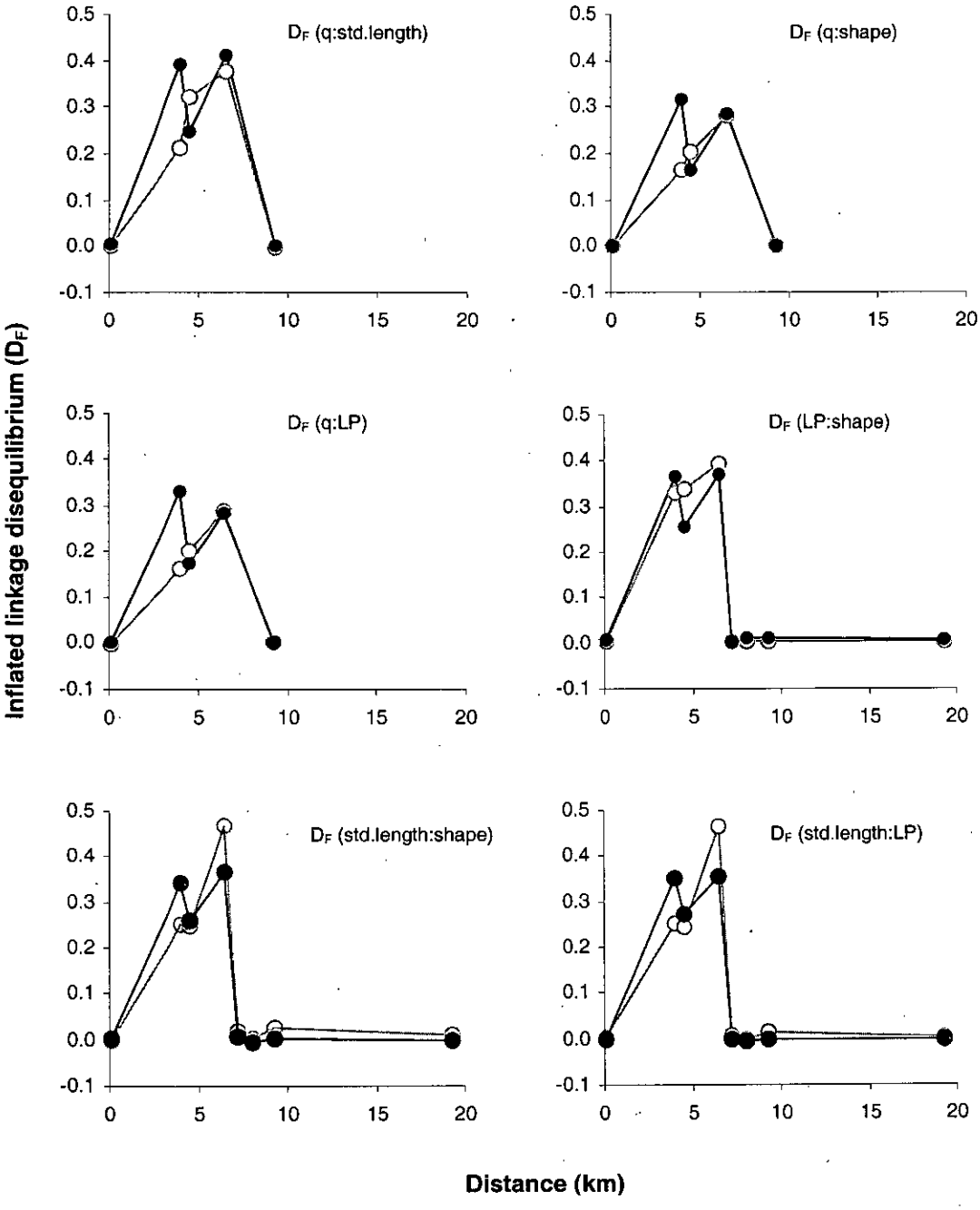


Figure 4.5. Estimates of linkage disequilibrium (D_F) between quantitative traits (specified in parentheses) across the hybrid zone. White circles represent females, solid circles represent males. Site 1 (distance = 0km), Site 8 (distance = 19.3km).

ESTIMATES OF DISPERSAL AND STRENGTH OF EFFECTIVE SELECTION

The estimate of the dispersal rate per generation (σ) we obtained from the maximum observed linkage disequilibrium within the hybrid zone (R, Site 3 = 0.313) was 1.21km x $gen^{-1/2}$ (0.90km x $gen^{-1/2}$ – 1.57km x $gen^{-1/2}$). Due to the low confidence we have in the estimated cline widths for bAR2 and MyoHC, and the lack of fixed differences in allele frequency at either end of the cline at these two loci, our calculation of σ was based on average cline width of the loci ATP1a2 and Cytb (6.11km) and the widest of their support limits (4.57km – 7.95km). Owing to the specific assumptions made here, and those general to this approach (mentioned above, see Methods) this estimate of dispersal rate per generation should be treated with caution. Our mark-recapture field methodology enables us, to some degree, to resolve the reliability of this dispersal estimate. During the period of April – December 2003, we tagged and released individuals on a monthly basis from each of the eight sites across the hybrid zone to determine the extent of within-stream movement (Jones *et al.* in prep). Of the 3,500 individuals we tagged, approximately 10% were recaptured, but only 1 of these individuals moved between sites (site 2 to site 3; a distance of 0.5km). Put into context, a movement from site 2 –3 represents the shortest distance between any of our sampling sites, but at that time (September 2003) the population size of fish in Site 2 was the also lowest of all riverine collection points (estimated at 2,200 fish at Site 2). A single recording of a fish moving between these sites corresponds to a minimum crude estimate of 100 fish based on tagged/untagged ratios. This stretch of river becomes a single homogenous pool at high tide, thus further facilitating movements between these sites. Our field estimate of dispersal has two limitations. Firstly, we only tagged individuals greater than 25mm standard length due to the mesh size of our traps and, therefore, could not detect dispersal of juveniles smaller than this size. Secondly, sudden dispersal

events triggered by environmental conditions (e.g. anadromous migration out to sea or into freshwater) may have fallen between our sampling periods. Therefore, our field observations probably underestimate the amount of dispersal that occurs in this system. In contrast, the estimate of dispersal based on linkage disequilibrium is likely to be inflated due to assortative mating and/or selection against hybrid fry. Therefore, the true level of dispersal probably lies somewhere between these two estimates. On this basis we believe the estimate of dispersal based in linkage disequilibrium is not unreasonable. Using our estimate of dispersal, we calculated the effective selection pressure (s*) to fall between 0.173 and 0.578. This indicates that fairly strong selection against hybrids would be required to maintain a cline of the width observed assuming the hybrid zone was a single locus system. Our confidence in the s* estimate is subject to the reliability of the dispersal estimate and additional assumptions (mentioned above – see methods) and should be interpreted with care.

ANALYSIS OF HETEROZYGOTE DEFICIT

F_{IS} values estimated for samples from sites 2-4 from cohort 1 were significantly larger than zero in all cases, except for juveniles collected from site 3. In no case did the estimates of F_{IS} for samples collected from site 7 differ significantly from zero. We found a marginally significant increase in F_{IS} over winter in cohort 1 (difference in average F_{IS} before and after winter = 0.058, p value = 0.063, Figure 4.6), although our power to detect this was low due to the small number of samples. In contrast, F_{IS} values in juveniles from cohort 2 showed a significant decline from July through to December 2003. The slope estimate from a regression of F_{IS} in sites 2-4 against time was significantly less than zero (slope = -4.12×10^{-4}, $\pm 1.46 \times 10^{-4}$ SE, p=0.014) and did not differ significantly between the three sites ($F_{2,10}$=0.226,

p=0.8019). F_{IS} values in site 7 did not change significantly with time ($2.36x10^{-4}$, ± $2.16x10^{-4}$ SE, p=0.340).

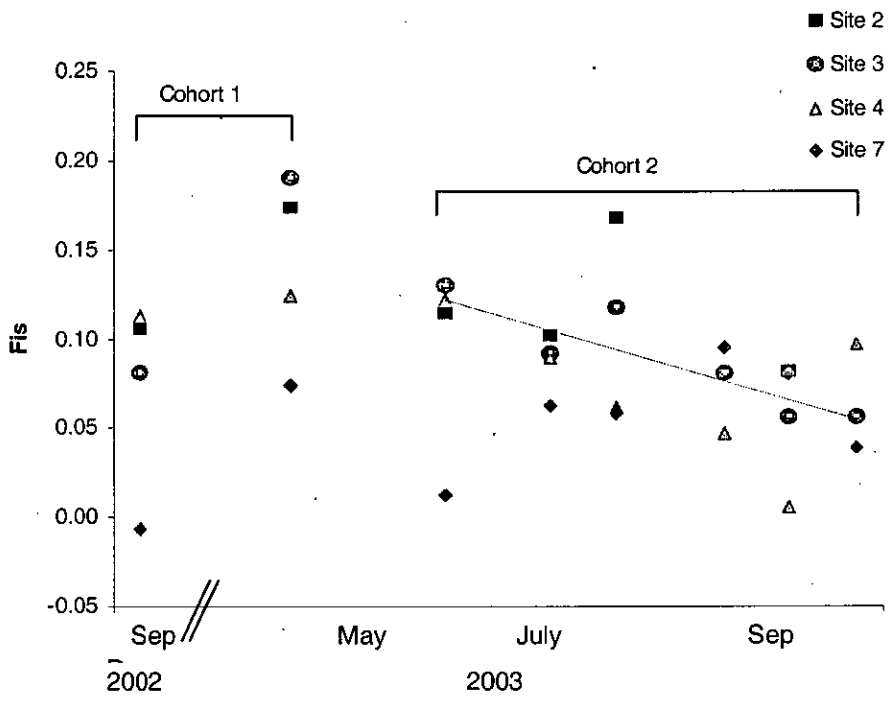


Figure 4.6. Estimated F_{IS} values for samples collected from cohorts 1 and 2. The line represents the change in F_{IS} over time in samples from sites 2-4 (p=0.014). The change in F_{IS} over time in samples from site 7 was not significant.

Temporal changes in F_{IS} are likely to be affected in part by the migration of anadromous juveniles out to sea. Examination of the distribution of q in individuals from cohort 2 in sites 2-4 revealed a sharp decline in the frequency of anadromous juveniles from August to September 2003 (Figure 4.7b) and a more gradual decline in the frequency of both anadromous and freshwater juveniles from September to December. In contrast, individuals of intermediate q remain at low frequency from July to December. Consistent with the observed increase in heterozygote deficit in cohort 1, we observed a decrease in individuals of intermediate q over winter (Figure 4.7a).

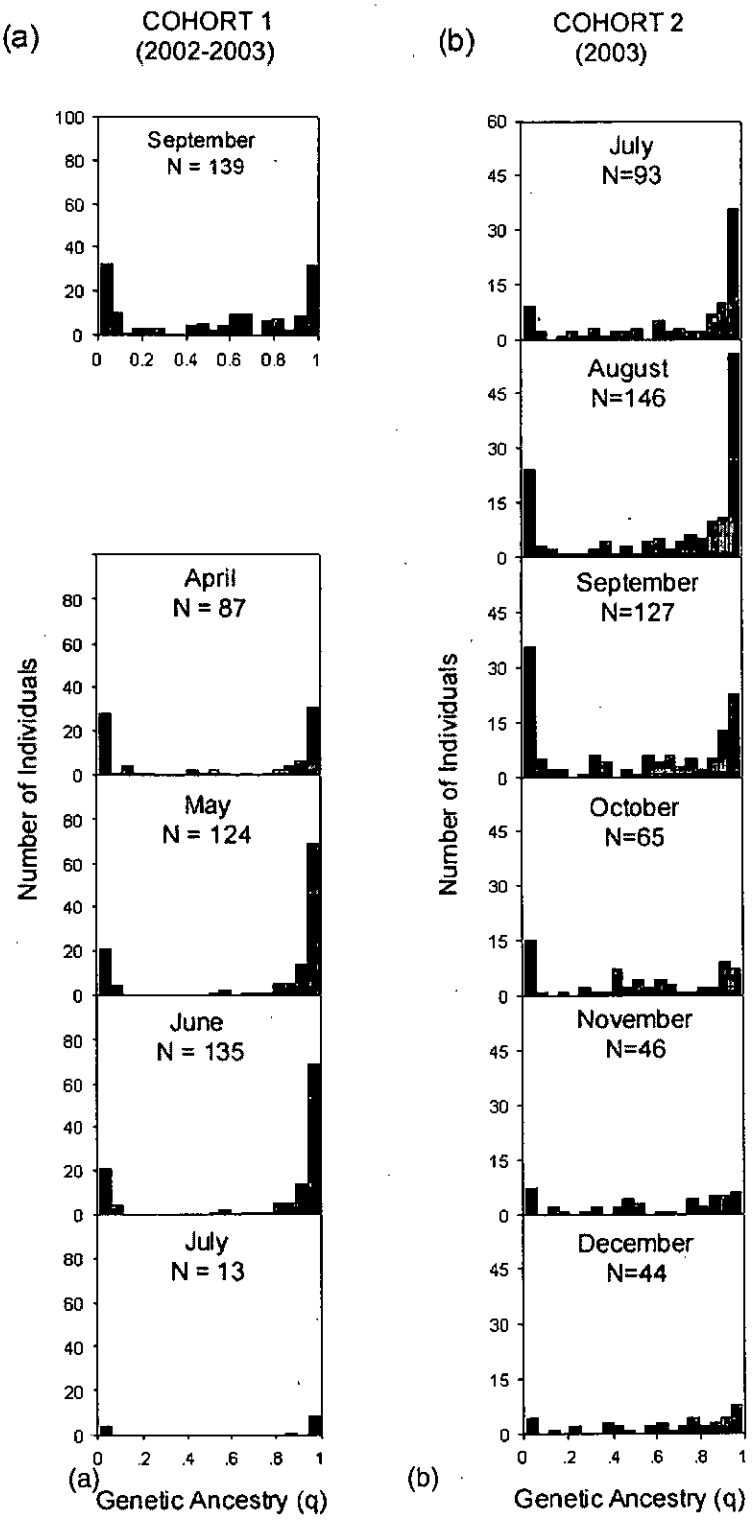


Figure 4.7. Distribution of q in cohort 1 (a) and cohort 2 (b) from sites 2-4 over time. q = 0 (freshwater), q = 1 (anadromous). N represents sample size.

FITNESS ANALYSES

We found a good correlation between q and LP within the hybrid zone (R^2 = 0.369, Figure 4.8), but independent variation in each trait is apparent. By including both q and LP as explanatory variables in the same models, we were able to assess the association between each of these traits and fitness independently.

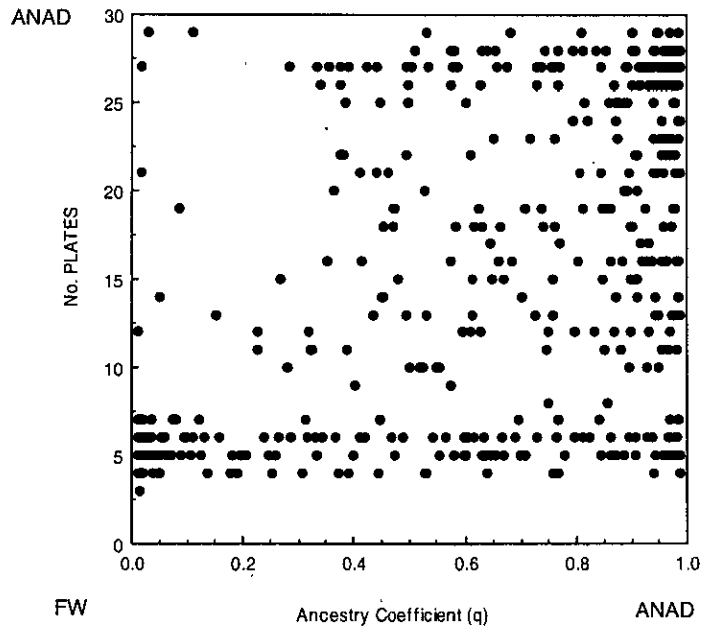


Figure 4.8. The relationship between the number of lateral plates (LP) and genetic ancestry coefficient (q) in juveniles from sites 2-4 (R^2 = 0.369). Axes are labelled with freshwater (FW) and anadromous (ANAD) extremes.

Juvenile standard length:

We found significant associations between juvenile standard length and both *q*, and LP and both these terms interacted significantly with other variables in the model. The LP^2 term explained a significant amount of variation ($F_{2,511}$=5.063, p=0.007), but the q^2 term did not ($F_{2,508}$=0.907, p=0.404) and was therefore removed.

The estimated coefficient for q was significantly less than zero (estimated coefficient -13.99 ± 2.70, $F_{1,508}$ = 56.23, p<0.001, Table 4.2), indicating that juveniles with freshwater ancestry were larger than individuals with anadromous ancestry. This is most likely caused by the earlier commencement of the freshwater breeding season (Chapter 3). The q term interacted significantly with day (estimated coefficient 0.037 ± 0.010 SE, $F_{1,508}$ = 13.29, p<0.001) revealing that the difference in standard length between freshwater and anadromous juveniles diminishes later in the year. A *post hoc* test revealed no relationship between q and standard length in juveniles sampled in the months of October – December ($F_{1,152}$=0.220, p=0.640) indicating that the asymptote point of pre-winter growth in these fish does not vary with q.

We detected a significant interaction between LP^2 and sex (Table 4.2, Figure 4.9). Females had a significantly positive LP^2 coefficient (0.012, ± 0.005 SE, p=0.006), indicating an association between individuals of intermediate plate number and small standard length (Figure 4.9a). The male LP^2 coefficient was significantly different to that for females (difference = -0.0189, ± 0.0065 SE, t_{508}= -2.9115, p = 0.0038) but not significantly different from zero (*post hoc* test: slope = -0.0056, ± 0.0050 SE, t_{508}=-1.1186, p=0.2644, Figure 4.9b). The significance of the quadratic term in females, but not in males, was also apparent in quadratic models performed on each sex separately (not shown). We used differentiation of the calculated coefficients to determine if the quadratic minima in females fell within the range of our data. In females, the minimum occurred when the number of plates was equal to 13.907, roughly mid-way in the plate number range (min 3, max 29).

Table 4.2. ANOVA Table for the minimised linear model of juvenile standard length in cohort 2 (N=517).

Term	Estimated Coefficient	+/- Standard Error	F ratio	Df	P value	
Intercept	11.30	2.06				
Sex	-3.83	1.29	1.56	1	0.213	
Day	-0.03	0.01	1.20	1	0.273	
q	-13.99	2.70	56.24	1	**<0.001**	***
LP	-0.34	0.15	7.39	1	**0.007**	**
LP^2	0.01	0.00	1.00	1	0.318	
q*Day	0.04	0.01	13.29	1	**<0.001**	***
Sex*LP	0.63	0.21	0.24	1	0.624	
Sex*LP^2	-0.02	0.01	8.48	1	**0.004**	**
Residual Mean SS			13.28	508		
Proportion of Variation explained by model:				16.18%		

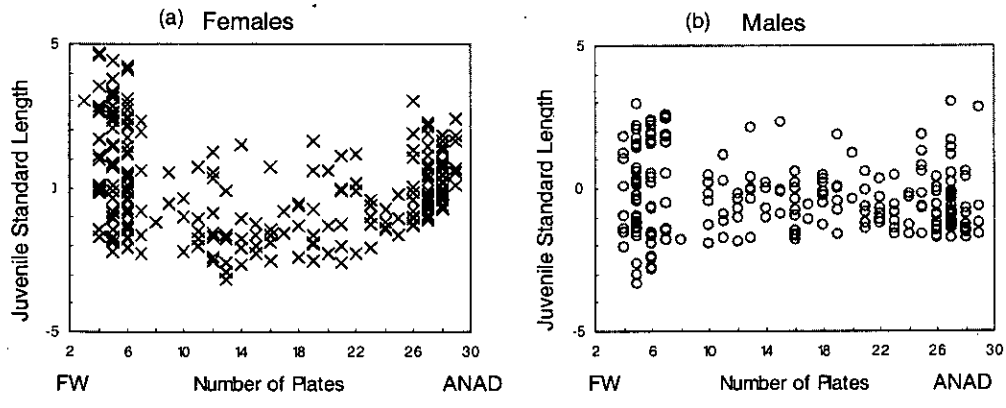


Figure 4.9. The predicted relationship between juvenile standard length and lateral plate number (LP) in females (a) and males (b). LP axis is labelled with freshwater (FW) and anadromous (ANAD) extremes.

Overwinter survival:

We found a significant quadratic association between overwinter survival and q and a marginally significant quadratic association with LP (Table 4.3). The estimated coefficient for the q^2 term was significantly greater than zero (q^2 coefficient = 10.758, ± 1.546 SE, t=-6.962, p<0.000). Individuals of intermediate q were less likely to reach adulthood than those of more extreme q scores (q minima determined from differentiation of coefficients = 0.455). We also found a marginally significant LP effect and an interaction between Sex and LP^2 (Table 4.3). The estimated LP^2 coefficient in females was larger than males (difference = -0.008, ± 0.005 SE, t_{482}=-1.815, p = 0.070). The LP^2 coefficient estimated for females was significantly larger than zero (0.006, ± 0.004 SE, t_{482}=1.682, one tailed p=0.047), but was not significantly different from zero for males (*post hoc* test coefficient = 0.003, ± 0.004 SE, t=0.787, two tailed p=0.432). The minima of the quadratic LP function for females coincided with a value of 12.5 plates, suggesting an association between reduced probability of overwinter survival and intermediate plate number in females. A surface plot of predicted values from the model shows that females with the lowest likelihood of reaching adulthood are those of intermediate LP and intermediate q (Figure 4.10). In comparison, the survivorship of males was influenced by q but not LP.

Table 4.3. ANOVA Table 4.for the minimised generalised linear binary model of overwinter survival in cohort 1 (N=490).

Term	Estimated Coefficient	+/- Standard Error	F ratio	Df	P value	
Intercept	1.49	0.58				
Sex	-0.96	0.81	0.01	1	0.930	
q	-9.79	1.57	3.43	1	0.065	
q^2	10.76	1.55	45.44	1	**<0.001**	***
LP	-0.15	0.11	3.64	1	0.057	
LP^2	0.01	0.00	0.31	1	0.578	
Sex*LP	0.25	0.15	1.00	1	0.318	
Sex*LP^2	-0.01	0.00	3.23	1	**0.073**	
Residual Mean SS			1.02	482		
Proportion of deviance explained by model:				16.28%		

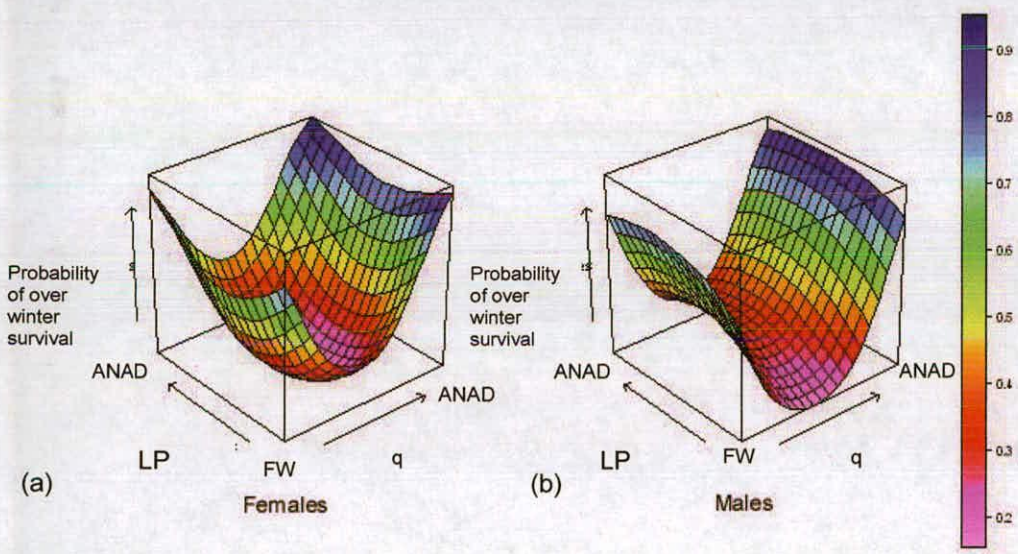


Figure 4.10. Association between overwinter survival and LP and q in females (a) and males (b) from cohort 1. Predicted surface plots from generalised linear logistic model. Axes are labelled with freshwater (FW) and anadromous (ANAD) extremes.

Female gravidity:

We found no main effect of LP, q, day or standard length on the probability of an adult female being gravid (Table 4.4). The q^2 term did not significantly improve the fit of the female reproductive fitness model (change in deviance = -2.025, 2df, p=0.363) and was removed, whilst the LP^2 term did (LP^2 change in deviance=6.891, 2df, p=0.032). We found significant two-way interactions between standard length and day, q and day, and LP^2 and standard length (Table 4.4). Large females were more likely to be gravid earlier in the breeding season whilst small females were more likely to be gravid later in the breeding season. Early in the breeding season q showed little association with the probability of a female being gravid. This is different later in the breeding season, when anadromous individuals were more likely to be gravid. LP was associated with the probability of an individual being gravid but this association changes depending on the size of the fish. In small fish, all LP morphs are equally likely to be gravid, but in large fish individuals of intermediate LP are less likely to be gravid than individuals of low or high LP (Figure 4.11).

Table 4.4. ANOVA Table 4.for the minimised generalised linear binary model of female gravidity (N=188).

Term	Estimated Coefficient	+/- Standard Error	F ratio	Df	P value
Intercept	-53.34	14.26			
Day	3.08	1.26	1.17	1	0.280
Std Length	-0.10	0.04	0.02	1	0.885
q	-20.70	5.44	0.02	1	0.882
LP	0.40	0.12	0.10	1	0.747
LP^2	1.17	0.29	0.00	1	0.965
q*Day	-0.05	0.02	15.26	1	**<0.001** ***
Std Length*Day	0.00	0.00	13.05	1	**<0.001** ***
Std Length*LP	-0.01	0.00	0.04	1	0.843
Std Length*LP^2	0.21	0.05	6.14	1	**0.014** *
Residual Error			0.99074	178	
Proportion of deviance explained by model:				11.03%	

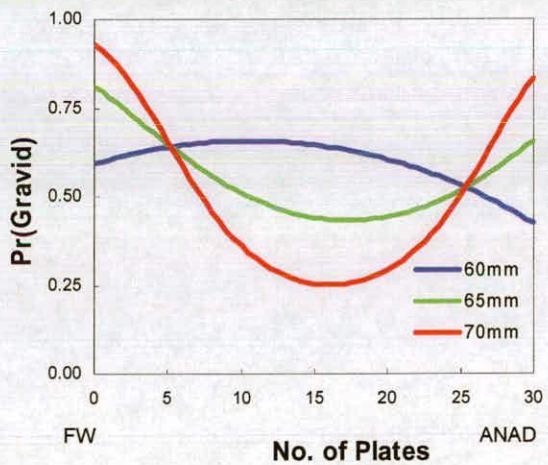


Figure 4.11. The quadratic relationship between LP and the probability of a female being gravid is dependent upon standard length. The predicted relationship is depicted here for three different standard lengths (mm). LP axis is labelled with freshwater (FW) and anadromous (ANAD) extremes.

DISCUSSION

The contact zone between anadromous and freshwater sticklebacks in the River Tyne is characterised by clines in allele frequencies and quantitative traits. Despite all nuclear markers being positioned in non-coding regions, we observed differences in the extent of differentiation of allele frequencies between anadromous and freshwater sticklebacks. At the ATP1a2 locus we found almost fixed differences in alleles between the two source populations whilst allele frequencies at the MyoHC and bAR2 loci differed by approximately 40 and 20 percent respectively. These differences between loci may be explained by neutral genetic drift or alternatively, by differences in the extent of linkage to genes under selective pressure. In other fish species (e.g. Zebra fish *Danio rerio,* and Atlantic salmon *Salmo salar*) the ATP1a2 gene codes for a Na+/K+ ATPase which in known to play a role in ion regulation in the cell (Shu *et al.* 2003). It is likely that this gene is under stronger divergent selective pressure than the MyoHC gene and bAR2 gene in anadromous and freshwater sticklebacks due to difference in the salinity of the habitats they occupy.

We observed fixed differences in haplotype frequencies at the mitochondrial Cytb locus and a significant upstream displacement in the centre of the mtDNA Cytb cline compared to those of nuclear markers. Previous studies of hybrid zones have also found displacement of mtDNA clines from the location of clines in nuclear genes (Ferris *et al.* 1983, Marchant 1988, Marshall *et al.* 2001) and this phenomenon often occurs in narrow hybrid zones (Harrison 1990). The upstream displacement of the mtDNA Cytb cline might be an indication of the historical location of cline, or, in an environmentally determined hybrid zone, a result of differences in the location of environmental gradients determining fitness. In our system, it may be linked to the

building of the weir above site four in the early 19th century, or historical changes in the position of a salinity gradient.

Variation in cline width is characteristic of hybrid zone models that invoke environmentally-determined selection pressures or selection against hybrids (Hewitt 1988). We found the cline in standard length to be significantly narrower than the clines in LP and shape. Lack of concordance in width (or shape) might be caused by differences in the number of loci underlying traits, the genetic determination of traits (e.g. additive or dominant), and the degree of linkage to other selected loci. A narrower cline would be expected in a quantitative trait controlled by fewer loci. Consistent with this idea, in a QTL map based on a cross between anadromous and freshwater sticklebacks, Colosimo *et al.* (2004) found a significant association between standard length and a single QTL, and an association of LP with a single QTL of major effect and 4 QTLs of minor effect. It is also plausible that standard length is under greater direct selective pressure than LP in this system. McKinnon *et al.* (2004) found a strong association between body size and assortative mate preference in anadromous and freshwater sticklebacks and argued that assortative mating based on size explains more variation in mate compatibility than assortative mating based on LP. We found only weak evidence for size-assortative mating between anadromous and freshwater sticklebacks from the River Tyne (Chapter 2), but it is possible that this may be stronger in the wild (Chapter 3).

We observed significant linkage disequilibrium and heterozygote deficit within the hybrid zone. Linkage disequilibrium appears to be generated by the presence of individuals of parental genotypes in the zone (as is evident in Figure 4.7). In this sense, the hybrid zone is bimodal (Jiggins and Mallett 2001) with bimodality being

strongest in samples of juveniles approximately 2-3 months old and in breeding adults. Continued immigration can generate sustained cytonuclear disequilibrium even in the absence of assortative mating (Arnold *et al.* 1988). In a previous paper (Chapter 3), we inferred that the observed heterozygote deficit in juveniles might be an indication of assortative mating in the wild. However, the lack of significant heterozygote deficit at some of the loci could equally be interpreted as evidence for postzygotic selection against hybrids at the early fry stage. We estimated that an effective selection pressure against heterozygotes of between 17% and 58% would be required to maintain the observed genetic cline width between anadromous and freshwater sticklebacks in the River Tyne. In our view, the assumption that D is due entirely to migration is the weakest point in our analysis. Thus, our estimate of an effective selection pressure may be an overestimate and should be treated with caution.

Does selection against hybrids occur inside or outside the hybrid zone? In a hybrid zone maintained by environmental gradients, parental genotypes may show lower fitness compared to hybrids due to the intermediacy of the environment (Moore 1977). It could be argued that the decline in heterozygote deficit over time provides evidence for hybrid superiority in juveniles within the hybrid zone. This trend might be due, in part, to an increase in the relative number of hybrids within the zone corresponding to the migration of anadromous juveniles out to sea. The noticeable decline in juveniles with anadromous q between August and September is consistent with other studies that have reported anadromous migrations occurring at 2-3 months of age (Bell and Foster, 1994). However, we also observe a decrease in the frequency of freshwater juveniles in our samples. What is the cause of this decline? We propose two possible explanations. The decline in the frequency of

juveniles with freshwater ancestry might be a direct result of mortality. This hypothesis implies hybrid superiority and requires a fresh influx of freshwater individuals into the hybrid zone for the next breeding season to restore the distribution of q to the bimodality observed in adults. Alternatively, the observed decline in freshwater juveniles might result from a switch in behaviour due to winter conditions. At the onset of winter, fish in cold climates enter torpor characterised by reduced activity levels (e.g. smelt *Osmerus eperlamus,* Vinni *et al.* 2005, Atlantic salmon *Salmo salar*, Harwood *et al.* 2002) and in some species switch to nocturnal behaviour (e.g. *Salmo salar,* Harwood *et al.* 2002, Hiscock *et al.* 2002). Hiscock *et al.* (2002) suggest that individual variation in activity patterns of juvenile Atlantic salmon (*Salmo salar*) during winter are, in part, driven by body size. Smaller fish forage more, presumably to decrease the risk of starvation. Increased activity during winter has been shown to reduce the probability of survival primarily due to the poor trade-off between low prey availability and energetic costs of foraging (Metcalfe *et al.* 1999). If such changes in behaviour are under genetic control then it is possible that hybrid individuals do not switch behaviour and are sampled more frequently in our traps during the winter months. This hypothesis implies hybrid inferiority.

We favour the latter hypothesis for several reasons: Firstly, in this river, our mark-recapture study (Jones *et al.* in prep) revealed high site philopatry in sticklebacks, and whilst the migration of large numbers of anadromous fish into the hybrid zone to breed can be expected, the migration of equally large numbers of freshwater fish seems implausible. Secondly, we have evidence of reduced fitness of recombinant juvenile phenotypes. Juvenile females of intermediate LP had smaller standard length than females of extreme LP score. Thirdly, if selection did not occur against

hybrids within the hybrid zone, then we must infer that it acts against hybrids outside the hybrid zone. This is plausible if hybrid juveniles adopt an anadromous migration strategy since the ocean environment differs significantly from the hybrid zone environment and recombinant phenotypes may be less fit in many ways. If, on the other hand, hybrids adopt a freshwater lifestyle, their movement upstream to freshwater locations is likely to be prevented by the existence of the weir immediately above site four. Therefore, for selection against hybrids to operate solely outside the hybrid zone, we argue that all hybrid individuals would need to undertake an anadromous migration – and this is unlikely.

Our fitness models take us some way towards understanding the nature of selection operating in this system. We found that there is independent variation in LP and q and this variation is likely to result from the genetic determination of LP. In two independent QTL analyses of LP, the same single, major QTL locus was identified (Colosimo *et al.* 2004, Cresko *et al.* 2004) and 3 minor "modifier" loci have also been identified (Colosimo *et al.* 2004). There is some evidence that the same genes are associated with this trait in different North American populations (Colosimo *et al.* 2004, Cresko *et al.* 2004). An individual of freshwater ancestry (as calculated by q) might possess an anadromous allele at a single locus of large effect that causes a large deviation from the predicted relationship between LP and q. In this sense, our measure of q reflects a genome wide measure of genetic ancestry, and LP a single trait controlled by one major locus. We discuss the associations between indirect measures of fitness and LP and q separately below.

Genetic ancestry (q) and fitness

Unlike Hagen (1967) we found evidence for hybrid inferiority in the form of reduced probability of overwinter survival in individuals with intermediate genetic ancestry. Overwinter hybrid inferiority might result from selection acting on individuals of intermediate q either inside or outside the hybrid zone, or both. In contrast, intermediate q was not associated with smaller juvenile standard length, since we found a negative linear (rather than quadratic) relationship. Temporal differences in breeding season of freshwater and anadromous sticklebacks is the best explanation for this negative relationship. Individuals of freshwater q start to breed earlier than individuals of anadromous q, although there is still considerable temporal overlap (Chapter 3), and in early samples of the cohort we found freshwater juveniles to be of larger standard length than anadromous juveniles. The negative relationship between q and juvenile standard length was dependent upon date of collection and decreased in strength towards the end of the year. This is evidence for the growth of juveniles asymptoting at the same size pre-winter. Of those individuals with intermediate q surviving to breed, we found no evidence of hybrid inferiority or superiority with regards to female reproductive fitness. Individuals of intermediate q were just as likely to be gravid as individuals of anadromous or freshwater q. In the absence of strong prezygotic isolation (such as variation in microhabitat nest location or assortative mating), backcrossing to freshwater or anadromous individuals is likely. Based on our analyses of fitness at three different stages of ontogeny, our findings suggest that the strongest selection against individuals of intermediate q occurs over winter.

Lateral plate number and fitness

We found evidence for reduced fitness of females of intermediate LP, independent of q, at all three stages of ontogeny. Females of intermediate LP were associated with small standard length in juveniles, reduced probability of overwinter survival, and of those surviving winter, larger females with an intermediate LP had a reduced probability of being gravid during the breeding season. Reduced fitness was not detected in males of intermediate LP (in the juvenile and overwinter models), therefore, the mechanism resulting in the observed association between LP and reduced fitness must involve a sex bias. We were not able to test whether intermediate LP affected male reproductive fitness within the scope of this study. We hypothesise that factors affecting the standard length of juveniles with intermediate LP in this system have knock-on effects on overwinter survival and reproductive fitness. We propose that the key to understanding the maintenance of the morphological cline in lateral plate number is the association between lateral plate number and juvenile body size.

We hypothesise that small juvenile standard length is due to reduced foraging or growth efficiency (or differences in energy investment) rather than intermediate LP directly. There are numerous possible traits that could be involved, including foraging behaviour (Mackney and Hughes 1995), foraging morphology, parasite resistance, energy uptake and growth, and differential life history investment. To explain our results, differences in these traits must show a sex bias and an association with LP independent of genetic ancestry. Sex differences in diet and niche-use have been reported in other stickleback populations (Reimchen and Nosil 2001) and might cause differential exposure to predators, parasites (Reimchen and Nosil 2001), environmental stressors or involve different metabolic costs for males

and females. Developmental instability might explain the smaller juvenile body size we observed because it is associated with increased prevalence of parasitism (Reimchen 2001) and individuals with high parasite load are likely to have lower energy resources for growth (Barber *et al.* 2001). In Boulton Lake, females with asymmetric plate patterns showed higher levels of total parasitism and increased frequencies of multiple nematode and multiple *Bunodera* infection relative to symmetric females but these effects were not apparent in males (Reimchen 2001). The increased parasitism was correlated with differences in diet but could also be explained by decreased immunocompetance (Reimchen 2001). To investigate these possibilities, studies of diet and parasite load are currently being carried out in the River Tyne hybrid zone.

Although we propose that factors affecting foraging or growth are important for maintaining the morphological cline in LP, this does not exclude the role of predation as a selective agent acting on LP inside or outside the zone of sympatry. Posterior plates of completely plated morphs reduce the probability of ingestion by piscivores (Reimchen 2000), which are common in the open waters of the Atlantic Ocean (Reimchen 2000). A lack of plates may increase the chances of surviving attacks by wading birds (Reimchen 1994), which are more of a threat in shallow inland waters. Therefore, it is likely that individuals of intermediate LP are at a selective disadvantage in either environment, and predation is likely to contribute to the reduced overwinter survival probability that we observed. This effect cannot be strong, however, because it was not observed in males, and unless there are sex differences in niche-use in the oceanic phase, there is no reason to assume that predators would target females over males. Because our models show that females are under greater selective pressure than males, we might expect to find a male

biased sex ratio in adults. Unfortunately, we are unable to test this hypothesis with the current data set because males are territorial during the breeding season and are less likely to swim into our traps than females. Field-based studies of breeding behaviour and assortative mating are needed to verify this.

Why is female fitness more closely associated with LP variation than q variation? The genes controlling LP may be closely linked to genes of large effect on an individual's fitness. In support of the genic view of speciation (Wu 2001), genes with large effect on an individual's fitness and viability, so called "speciation genes", have been found in several organisms e.g. *Drosophila* (Ting *et al.* 1998), *pea aphids* (Via and Hawthorne, 2001). Genes affecting reproductive isolation are often located on the X chromosome (termed the "large X chromosome effect", Charlesworth *et al.* 1987), because advantageous recessive mutations will be expressed in the heterogametic sex. In sticklebacks, males are the heterogametic sex and the sex determining region lies on a different chromosome to the major LP locus (Colosimo *et al.* 2004, Peichel *et al.* unpublished). Genes affecting reproductive isolation might not cause a reduction in fitness in the context of their own genetic background, but can cause reduced hybrid fitness due to epistatic interaction effects with genes from different genetic backgrounds (Orr 1995). Speciation genes were closely linked on the same chromosomal region in the pea aphid (Via and Hawthorne, 2001) and it is therefore plausible in sticklebacks that LP is linked to a gene of large fitness effect which interacts with genes on the X chromosome. Our findings hint at the possible underlying genetic architecture of genes associated with speciation in sticklebacks.

CONCLUSIONS

In this paper, we have presented clines in morphology and genetics across a hybrid zone between anadromous and freshwater sticklebacks in the River Tyne. The position of the centre of the mitochondrial cline was significantly further upstream than the centre of clines at nuclear loci. We estimated the effective selection pressure (s*) required to maintain clines of the observed width to be between 17% and 58% and this is likely to represent the upper bounds since it is inflated by the presence of assortative mating or selection against hybrid fry. Morphological and genetic divergence is being maintained by selection against hybrids and recombinant phenotypes both within the hybrid zone and, it is likely, outside the hybrid zone. Although we observed an increase in the proportion of hybrid genotypes sampled over time in juveniles, we found that individuals of intermediate q were significantly less likely to be sampled after winter. We also found that females of intermediate LP were of smaller juvenile standard length, had lower probability of overwinter survival, and had decreased probability of being gravid relative to size. The association of intermediate LP, but not intermediate q, with female fitness might be explained by tight linkage between LP and genes of large fitness effect. Decreased fitness of females of intermediate LP might result from epistatic interactions between genes located on the X chromosome, and suggests that genes responsible for the divergence between anadromous and freshwater sticklebacks are closely linked. This is the first study of an anadromous-freshwater stickleback hybrid zone to show evidence of reduced hybrid fitness in the wild.

CHAPTER FIVE

A candidate gene association study in a stickleback hybrid zone

ABSTRACT

Do the same genes underlie variation in the same traits in different populations? The threespined stickleback *(Gasterosteus aculeatus)* is a model organism for studies of the evolution of quantitative traits due to the enormous efforts put into constructing linkage maps and unravelling the genetic architecture underlying traits. One question of interest is whether variation in a given trait in two different populations involves the same or different genes. We investigated associations between alleles and traits in wild sticklebacks from an anadromous–freshwater hybrid zone in the River Tyne, Scotland, using seven candidate loci identified as being linked to traits in sticklebacks from Priest Lake, Canada, plus three additional nuclear markers and a mitochondrial marker. With both a case-control approach and linear models, we explored how variation in spine lengths and lateral plate number was associated with the candidate markers. We investigated associations within both an anadromous and a freshwater stickleback population, as well as a sample of individuals identified as hybrids. Here, we report our findings of statistical associations between alleles and traits, some of which were consistent with linkage studies in other stickleback populations. These consistencies suggest that similar genes may contribute to variation in the same traits in different stickleback populations. However, we also found several associations between loci and traits that differ from those reported in other studies, suggesting possible differences in the genetic determination of traits may also exist. The associations between traits and markers found in hybrids must be interpreted with caution since the presence of linkage disequilibrium within these individuals will cause spurious associations to be detected.

INTRODUCTION

The existence of variation in quantitative traits within and between species is interesting to evolutionary biologists because it provides insight into how adaptation to specific environments and, ultimately, speciation occurs. Identifying genes that underlie variation in traits is of particular interest because they code the heritable component of variation on which selection acts and enables us investigate questions such as; how many genes are involved in the evolution of trait? Are there many genes of small effect, or few genes of large effect? Do the same genes underlie trait variation in different populations? Are different genes affecting a trait closely linked? What kind of mutations result in trait variation (e.g. coding or regulatory region?). Mapping the location of quantitative trait loci (QTLs) is becoming increasingly common and several studies have already provided insights into the questions listed above (e.g. Peichel *et al.* 2001, Colosimo *et al.* 2004, Cresko *et al.* 2004).

The threespine stickleback *(Gasterosteus aculeatus)* is proving to be a model natural organism for the study of quantitative trait loci, due to both its evolutionary history and the enormous efforts put into understanding its genome (e.g. Peichel et al., 2001, Colosimo *et al.* 2004, Cresko *et al.* 2004). Since the retreat of the last ice age, approximately 20,000 years ago, the stickleback has undergone an adaptive radiation with multiple invasions of freshwater habitats by marine sticklebacks. Throughout the northern hemisphere, independent and parallel divergence of the same morphological traits (e.g. reduction of lateral plate morphology, spine length, and pelvic girdle), has occurred as a result of adaptation to the freshwater environment (McKinnon and Rundle 2002). The fact that parallel evolution has

occurred in such a short time makes sticklebacks particularly useful for studies of quantitative trait loci because, unlike many other organisms, the divergence in morphology is not accompanied by vast genome wide divergence. In addition, repeated invasions have sometimes led to the formation of reproductively isolated species pairs within lakes (e.g. Canada, McPhail 1995).

Peichel *et al.* (2001) laid the foundations for the utility of the stickleback for studies of the evolutionary genetics of quantitative traits when they constructed a linkage map from a cross between benthic and limnetic sticklebacks from Priest Lake, British Columbia, Canada. F1 progeny from this cross were used to identify major QTLs underlying several traits including the number of lateral plates, the length of 1st and 2nd dorsal, and pelvic spines amongst others. In this cross, more than one QTL was identified as affecting lateral plate number, 1st, and 2nd dorsal spine length, and each of these QTLs were located on different linkage groups, (except that one of the QTLs affecting 2nd dorsal spine length and the QTL affecting pelvic spine length mapped to the same linkage group, Table 5.1). Peichel *et al.'s* (2001) study suggested that genes of major effect rather than many genes of small effect may underlie phenotypic variation in sticklebacks, and set up the question of whether the same QTL underlie variation in the same traits in different populations.

Table 5.1. A summary of stickleback QTLs identified to date. ** Pelvic armour reduction refers to the presence or absence of both the pelvic girdle and pelvic spines. *** Associated with the number of anterior lateral plates only. N.b. STN183 was originally mapped to a separate linkage group (LG XVIII), but in later crosses (Colosimo et al. 2004) this linkage grouped merged with linkage group IV (Cresko Pers. Comm.). Information on the distance between STN183 and Gac4174 is currently unavailable.

Trait	Author															
	(a) Piechel et al (2001)		(b) Cresko et al (2004)		(c) Colosimo et al (2004)		(d) Shapiro et al (2004)		Present Study							
									Anadromous		Hybrid		Freshwater			
	benthic x limnetic Priest Lake, Canada		*marine x freshwater (3 Alaskan pops)*		*marine (Japan) x freshwater (Canada) high plated x low plated (Friant Lake, California)*		*Same marine x freshwater cross as (c)*		*Wild population*		*Wild population*		*Wild population*			
	Locus	*Linkage Group*	*Locus*	*Linkage Group*	*Locus*	*Linkage Group*	*Locus*	*Linkage Group*	*Locus*	*Linkage Group*	*Locus*	*Linkage Group*	*Locus*	*Linkage Group*		
Lateral Plate Number	STN152	*LG XIII*	STN183	*LG IV*	Gac4174	*LG IV*			STN208	*LG XXVI*	STN208	*LG XXVI*	bAR2	unknown		
	STN208	*LG XXVI*			STN71	*LG VII*			STN152***	*LGXIII*	STN130	*LG XI*				
					STN124	*LG X*			STN9***	*LG I*	Cytb	mtDNA				
					STN219	*LG XXVI*			ATP1a2***	unknown	STN152***	LG XIII				
1st Dorsal Spine Length	STN9	*LG I*									Cytb	mtDNA				
	STN26	*LG II*														
2nd Dorsal Spine Length	STN96	*LG VIII*									STN94	*LG VIII*	STN130	*LG XI*		
	STN130	*LG XI*									Cytb	mtDNA				
Pelvic Spine Length	STN 94	*LG VIII*									STN94	*LG VIII*	STN152	*LG XIII*		
											Cytb	mtDNA				
Pelvic Armour Reduction **			STN82	*LG VII*			*Pitx1*	*LGVII*								

In two independent studies of recombinant progeny of crosses between different morphotypes, both Colosimo *et al.* (2004) and Cresko *et al.* (2004) found evidence that the same QTL region was associated with lateral plate morphology in different stickleback populations (Table 5.1). Colosimo *et al.* (2004) used a Japanese marine female and a Canadian, Paxton Lake, benthic male to generate F2 progeny using a backcross design. In this cross, they identified 4 lateral plate QTL, one of which (on linkage group IV) explained 77% of variation in plate number, and the remaining three (on linkage groups VII, X, and XXVI) explained between 3-5% of variation each. One of these minor lateral plate QTLs (linkage group 26) located close to one detected in the original Priest Lake cross (Peichel *et al.* 2001). In a second cross, Colosimo *et al.* (2001) established that three of the lateral plate QTLs identified in their first cross (including the major locus) were also affecting lateral plate morphology in sticklebacks from Friant Lake (California). A complementation study confirmed that it was the same major gene explaining variation in lateral plate number. At the same time, in a separate study, Cresko *et al.* (2004) used complementation studies and linkage mapping in a study of lateral plate morphs in three Alaskan stickleback populations and identified the same major QTL as Colosimo *et al.* (2004), underlying lateral plate morphology on linkage group four. Cresko *et al's* (2004) study also identified a major QTL located on linkage group seven associated with pelvic reduction. This finding was supported by the work of Shapiro *et al.* (2004) who showed that reduced expression of the candidate developmental gene, *Pitx1* (located on linkage group VII, Table 5.1), was associated with pelvic reduction. One pattern emerging from these studies is that the same genes of major effect (or group of genes in the same location) underlie variation in quantitative traits in different stickleback populations.

There is also evidence, however, for differences in genes of minor effect on quantitative traits. For example, the locus STN152 identified as being associated with lateral plate variation in Priest Lake sticklebacks (Peichel *et al.* 2001) was not associated with lateral plate number in the marine-freshwater cross by Colosimo *et al.* (2004). Similarly, the minor lateral plate QTL STN71 (Colosimo *et al.* 2004) was not associated with lateral plate number in the Friant Lake cross, although sample sizes in this cross, and therefore power to detect this association, was low. It is possible that QTL of minor effect may differ between stickleback populations.

In this study, we set out to determine if markers linked to quantitative traits in Peichel *et al.'s* (2001) original study, were also associated with quantitative traits in a natural freshwater-anadromous population of Scottish sticklebacks. Our study is distinct from a QTL mapping study because we do not aim to identify particular regions of the genome linked to particular traits. Rather, we test to see whether particular candidate markers, from the previous QTL study of Canadian lake fish (Peichel *et al.* 2001), show associations with traits in our study populations.

There are several different ways to test for associations between traits and loci, the most appropriate method depends on the study population (e.g. natural population or a laboratory cross) and the nature of the trait (qualitative or quantitative). For example, in a simple disease association study, individuals are genotyped at a number of markers and then tested for significant differences in allele frequencies between case (infected) and control (uninfected) groups (Pritchard and Donnelly 2001). A significant association would imply that the marker is closely linked to the disease locus since, in randomly mating populations, linkage disequilibrium decays

rapidly with genetic distance (Pritchard and Donnelly 2001). Alternatively, for a quantitative trait, linear models can be used to determine if the number of copies of an allele is significantly associated with the trait score. When allelic diversity is high, this approach requires large sample sizes to ensure each possible allele is represented in a large number of individuals. For this reason, linear models are most applicable to studies where allelic diversity is low such as those utilising laboratory crosses or bi-allelic markers.

The existence of population structure within an association study can result in high frequencies of spurious associations being detected (Pritchard *et al.* 2000). This is because the proportion of individuals of a given subpopulation may differ within the case and control samples, or within individuals with extreme quantitative trait scores. In addition to different frequencies of alleles at the underlying QTL and physically linked markers, the case and control samples may also differ in allele frequency at many other unrelated loci, thus resulting in "spurious" associations (Pritchard and Donnelly 2001). Using a valid statistical method when testing for association in the presence of population structure has, therefore, become a major concern in association studies (Pritchard et al 2000). Pritchard and Donnelly (2001) developed a two-step approach to deal with this problem. Firstly, the presence of population structure is determined using a Bayesian Montecarlo Markov Chain (MCMC) approach to cluster individuals based on multilocus genotypes (Falush *et al.* 2003). Secondly, the probability of a given individual having ancestry in the estimated clusters is used to 'correct' for the presence of structure in the association test (Pritchard and Donnelly 2001). An alternative approach to avoid difficulties of population structure in association studies, called the transmission/disequilibrium

test (TDT), was developed by Spielman *et al.* (1993). TDT studies compare genotypes of parents and offspring to detect associations between markers and traits and are therefore not susceptible to false postives even in the presence of population structure. Although genetic techniques and resources are becoming increasingly available, the requirement of pedigree information for TDT association studies (and other family based studies) is a major limiting factor for studies involving natural populations.

In this paper, we investigate associations in wild sticklebacks sampled from an anadromous-freshwater hybrid zone in the River Tyne, Scotland. The existence of recombinant individuals makes hybrid zones akin to natural laboratories (Hewitt 1988) and, therefore, suitable for studies of QTL involved in species divergence and speciation (Rieseberg and Buerkle 2002). The presence of pure source populations allows us to explore associations between markers and quantitative trait variation within freshwater and anadromous populations. In addition, the presence of hybrid individuals, with increased phenotypic variation, provides an opportunity to detect markers associated with between-species trait variation. We use a case-control approach to identify loci and alleles showing associations with traits and further explore these associations using linear models.

MATERIALS AND METHODS

Sample collection

Our analysis of association between traits and loci was performed on three different data sets. These data sets were comprised of sticklebacks sampled from three distinct sites in the River Tyne, East Lothian, Scotland and represent a subset of

sticklebacks sampled as part of a larger study of an anadromous-freshwater stickleback hybrid zone (Chapter 3, Chapter 4). The 'anadromous' data set consisted of individuals collected from rockpools at the mouth of the river (Site 1). Fish sampled from this site had characteristic anadromous morphology (Bell and Foster 1995, Chapter 3) and were identified as consisting of 100% 'anadromous' genetic composition (Chapter 3). Our 'hybrid' data set included individuals sampled from Site 4, located approximately 5.5 km upstream from Site 1. This site is a freshwater site and represents the approximate centre of the hybrid zone. Samples from this site showed a large amount of phenotypic variation (Chapter 3). For the purposes of this study we used individuals identified as 'hybrids' based on the probability of them having freshwater or anadromous genetic ancestry using the program STRUCTURE (Falush *et al.* 2003, Chapter 3). Individuals from Site 4 with 'freshwater' or 'anadromous' ancestry were excluded from the analysis. Our 'freshwater' data set was comprised of individuals collected from a freshwater site a further 5.5 km upstream (Site 7). Fish sampled from this site had freshwater morphology similar to that reported in many other freshwater stickleback populations (shorter spines, fewer lateral plates, more streamlined body shape, and olive colouration, Bell and Foster 1995). 95% consisted of individuals of 'freshwater' genetic ancestry (the remaining 5% identified as 'hybrids'). These hybrids were excluded from the data set.

Due to the design of our hybrid zone study, multiple samples from each site were collected over time, the majority of individuals being collected on a monthly basis during 2003 (see Chapter 3, Table 3.2). In this respect, individuals from Sites 4 and 7 represent four distinct cohorts and consist of both juveniles and adults. Individuals

from Site 1 were adults representing 2 cohorts. Freshwater sticklebacks in this system have a one-year lifespan (Jones *et al.* in prep.) and therefore non-overlapping generations, but the lifespan of anadromous sticklebacks remains unknown.

Fish were collected using wire mesh minnow traps placed overnight. Prior to April 2003, individuals were killed using a UK Home Office Schedule 1 method and preserved in ethanol. From April–December 2003 individuals were sedated, morphometric data collected (see below), fin-clipped, tagged with visible elastomer, and revived before being released back into the river. This procedure was performed under Home Office Licence (60/2954). Tagging enabled us to identify previously sampled individuals and avoid replication in our data.

Morphological Data

We collected data on the following morphological traits: length of first dorsal, second dorsal, and pelvic spines, lateral plate number and pattern on the left side of the body, and the presence or absence of a keel on the caudal peduncle (Figure 5.1). Morphological measurements were collected on live fish in the field, or ethanol preserved specimens in the laboratory using callipers accurate to 0.02mm (for spine lengths) and a seeker tool (for plate counts). Standard length was calculated from digital photographs of specimens against a 5mm background grid. To remove the differences in spine length due to growth we calculated residuals from an asymptotic non-linear regression of spine length against standard length: Spine Length = $a - b * e^{(-c * \text{Standard Length})}$ for each cohort and sex separately, where the coefficient a determines the asymptote, and b and c determine the slope. We use this simplified approach rather than the more popular von Bertalanffy growth model (1938)

because our samples do not represent repeated measures of the same individual. Residual spine length obtained from this regression was used in further analysis.

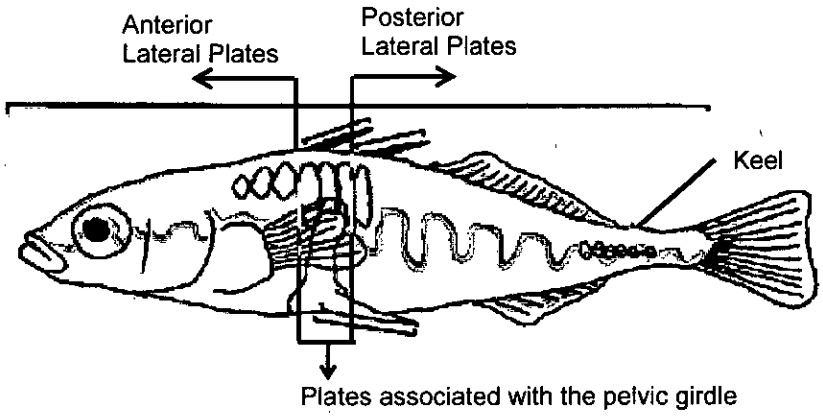


Figure 5.1. Trait measurements and lateral plate data collected from sticklebacks. This individual would have the plate pattern formula 1,3,3 (indicating the number of posterior, pelvic, and anterior plates respectively) and a total plate count of 7.

Lateral plate pattern was recorded using the following method, starting at the caudal peduncle and moving in an anterior direction. Firstly, the number and sequence of plates and gaps in plates posterior of the pelvic girdle was recorded. Then, the number of plates in association with the pelvic girdle, and finally, the number of plates anterior to the pelvic girdle were recorded (Figure 5.1). We observed almost no variation in the number of plates associated with the pelvic girdle (across all three data sets, only 4 individuals (0.005%) did not have a score of 3, see results Table 5.3). This variable was not used in further analysis. The number of anterior lateral plates and posterior lateral plates were correlated (R^2 = 0.392 (hybrid), 0.0041 (anadromous) and 0.1144 (freshwater)), however, the existence of independent variation justifies treating them as distinct variables. Furthermore, the study by

Cresko *et al.* (2004) suggests that anterior lateral plate formation may be governed by QTL independent of posterior lateral plate QTL.

Genotyping

Fish were genotyped at seven microsatellite markers linked to QTL underlying first and second dorsal and pelvic spine length and lateral plate morphology in a cross between limnetic and benthic sticklebacks from Priest Lake (Table 5.1) as identified by Peichel *et al.* (2001). At the time of genotyping, the additional studies of lateral plate pelvic reduction QTLs were not published so the new markers associated with traits by Colosimo *et al.* (2004), Cresko *et al.* (2004) and Shapiro *et al.* (2004) were not typed in this study. In addition, fish were genotyped at 3 SNPs located within introns of a Na^+/K^+ ATPase (ATP1a2 intron 1), myosin heavy chain (MyoHC intron 5) and beta androgen receptor (bAR2 intron 2) and a SNP located within the cytochrome b gene of the mitochondrial genome. These SNP's are located within functional genes associated with ion regulation (in salmon (*Salmo salar*) and zebrafish (*Danio rerio*), e.g. Shu *et al.* 2003), muscle protein (sticklebacks, McGuigan *et al.* (2004)) and hormone pathways (sticklebacks, Hellqvist *et al.* 2004) respectively, and were chosen based on the availability of sequence data from multiple fish species and the existence of polymorphism in River Tyne sticklebacks. A sex-linked indel located at the 3' untranslated region of the Isocitrate dehydrogenase gene (Peichel unpublished data) was used to sex each individual. DNA was extracted, PCR's and restriction enzyme digests performed as specified in Chapter 3.

Statistical Analysis

Overview

Associations between continuous traits and alleles can be detected using a linear model where the number of copies of each of the given alleles is entered as an independent factor. In a sample of individuals from a wild population, the utility of this approach is restricted by allelic diversity, since the large number of variables in the model would require a very large sample size. Reduced sample sizes result in an incomplete matrix. In this paper, we use a targeted method to identify candidate alleles which may be associated with each trait, and then perform a linear model to determine whether this select group of alleles explain variation in the trait of interest. Using χ^2 tests in a case-control association study framework (e.g. Pritchard and Donnelly 2001, Glorioso *et al.* 2001), we firstly targeted loci, then alleles at a locus, that differed significantly in frequency between individuals with extreme trait scores.

Testing for population structure within each data set:

Association studies are susceptible to false positives, firstly, due to the large number of tests performed, and secondly, due to the presence of population structure within the samples. We therefore tested for evidence of population structure within each of our data sets. Temporal samples for each data set were pooled and tested using the program STRUCTURE (Falush *et al.* 2003). STRUCTURE uses a Bayesian MCMC approach to cluster individuals on the basis of their multilocus genotypes without *a priori* information about population of origin. The likelihood of there being as many as *K* distinct genetic clusters within the sample can be estimated, and thus the most likely number of genetic clusters determined. STRUCTURE was run with a burn-in period of 100,000 replicates, followed by 1,000,000 replicates from which the

likelihood of there being *K* clusters was determined. This analysis was performed on the anadromous and freshwater data sets in a previous paper (Chapter 3) and was only performed on the hybrid data set here.

Case and control group classification:

We classified individuals into 'case' and 'control' groups by ranking individuals in the data set by trait score and pooling individuals in the upper third and lower third of the sample. Individuals with intermediate trait values were excluded from the analysis. For the purposes of this analysis, we considered total lateral plate number to be a continuous variable. Where the boundaries of the case and control thirds fell within the middle of a particular plate count category, we included all individuals with that category within the case or control group. The presence and absence of a keel is a binary measure which naturally falls into case and control groups. Individuals were also grouped by plate pattern and groups represented by more than 15 individuals were included in a test with multiple case and control groups. Under-represented plate pattern groups (less than 15 individuals) were excluded from the analysis. Details of the sample sizes in each of the case and control groups for each of the traits in each of the data sets are specified in Table 5.2.

	1st, 2nd Dorsal and Pelvic Spine Length		Total Number Lateral Plates		Number of Posterior Lateral Plates		Number of Anterior Lateral Plates		Keel		Plate Pattern	
	Criteria	*N*	*Criteria*	*N*	*Criteria*	*N*	*Criteria*	*N*	*Criteria*	*N*	*Criteria*	*N*
Anadromous Data Set	long spines	26	<28	34	<22	40	3	56	present	79	22,3,3	15
	short spines	26	>28	20	>22	39	4	23	absent	0	23,3,3	19
	excluded	27	excluded	25	excluded	0	excluded	0	test not performed		None of above	45
Hybrid Data Set	long spines	93	<8	109	<2	108	<3	110	present	206	0,3,1	19
	short spines	93	>19	110	>14	109	>3	110	absent	110	0,3,2	29
	excluded	93	excluded	97	excluded	99	excluded	96	excluded	0	1,3,2	28
	NA	37									21,3,3	26
											22,3,3	21
											None of above	193
Freshwater Data Set	long spines	144	<5	118	0	238	0 or 1	142	present	14	0,3,1	101
	short spines	144	>5	164	>0	178	>1	274	absent	430	0,3,2	33
	excluded	145	excluded	162	excluded	28	excluded	28	test not performed		0,3,3	16
	NA	11									1,3,1	102
											1,3,2	119
											1,3,3	15
											None of above	58

Table 5.2. Summary of criteria used to define extreme trait groups in each of the three data sets. NA = individuals with missing trait data, excluded = individuals of intermediate trait value, None of above = individuals who did not fall into the above-mentioned plate categories.

The program STRAT (Pritchard and Donnelly 2001) was used to test for differences in allele frequencies between case and control groups. In the absence of population structure STRAT performs an R x C χ^2 test for heterogeneity (where R represents the number of alleles at a given locus, and C the number of groups being compared) and returns the overall probability of differences in allele frequencies at a given locus between the C groups. Alleles with fewer than 10 copies are pooled by the STRAT program. Allele frequencies within each group were estimated using an expectation-maximisation (EM) algorithm and p values determined by 10,000 simulated tests per locus. Loci showing significant association with traits were identified as those with significant differences in allele frequencies between case and control groups.

Our targeted approach then involved identifying alleles contributing to overall significant differences in allele frequency within a locus. R x C χ^2 tests that were significant at the $\alpha < 0.05$ level were consolidated into 2 x C χ^2 tests, where the 2 allele groups represent the frequency of the allele of interest and the frequency of all other alleles pooled together. This procedure was repeated for each allele at a locus to identify those with significant differences in frequency between the groups.

Linear Models

For each trait, we investigated the amount of variation that could be explained by the select groups of alleles which differed significantly ($\alpha<0.05$) between extreme trait groups in a linear model. Linear models were performed on each of the entire data sets since, in this approach, inclusion of individuals of intermediate trait value would improve power. In each model, the number of copies of a given allele (0, 1 or 2)

was entered as an independent factor. We explored only main effects because the high frequency of rare genotypes excluded the possibility of investigating higher order interactions. Where the number of individuals with 2 copies of an allele was less than 10, individuals were pooled with individuals with 1 copy of the allele. In some models, two alleles at a locus were included. We argue this is valid, since at these loci there were multiple alleles, and the two alleles were essentially free to vary independently. Models were minimised by stepwise removal of alleles that did not explain significant amounts of variation. Our linear models assume an additive effect of alleles associated with the trait since the contribution of each allele to the trait is determined by the equation:

$$\text{Trait} \sim \beta_a * \text{Allele(a)} + \beta_b * \text{Allele(b)} + \ldots\ldots + \beta_z * \text{Allele(z)}$$

where a, b . . . z represent each of the different alleles included in the model and ß represents the estimated coefficient for each allele. Generalised linear models with binomial error structure were used to explore variation in traits falling into two categories (the number of anterior lateral plates in anadromous fish, and plate pattern in anadromous fish). To explore the fit of the model, we plotted the independent effects of each allele on the trait in addition to plotting the mean trait score for each genotype combination.

RESULTS

Trait Variation

Traits, and variation in traits, differed considerably between each of the data sets. This difference was particularly noticeable in lateral plate morphology. We observed virtually no overlap in total lateral plate number between individuals in the anadromous and freshwater data sets, whereas the range in plate number observed in the hybrid data set overlapped with that observed in both anadromous and freshwater individuals (Figure 5.2a-c). This pattern was similar when only posterior plates were examined (Figure 5.2g-i) but in contrast, the number and range of anterior lateral plates (0-4) overlapped between data sets (Figure 5.2d-f). Males in the freshwater data set had significantly more anterior lateral plates than females (mean difference 0.186, $t_{425} = 8.705$, p=0.003) but no other sex differences in lateral plate morphology were observed in any of the data sets. Of the 316 hybrid individuals sampled, we observed 114 different plate patterns, whilst only 27 and 8 different plate patterns were recorded in freshwater and anadromous fish respectively. In total, 34 plate patterns were common to more than one data set (Table 5.3). Differences in mean 1st and 2nd dorsal and pelvic spine lengths between freshwater, hybrid and anadromous fish were also apparent in larger individuals and were most pronounced in females compared to males (Figure 5.3).

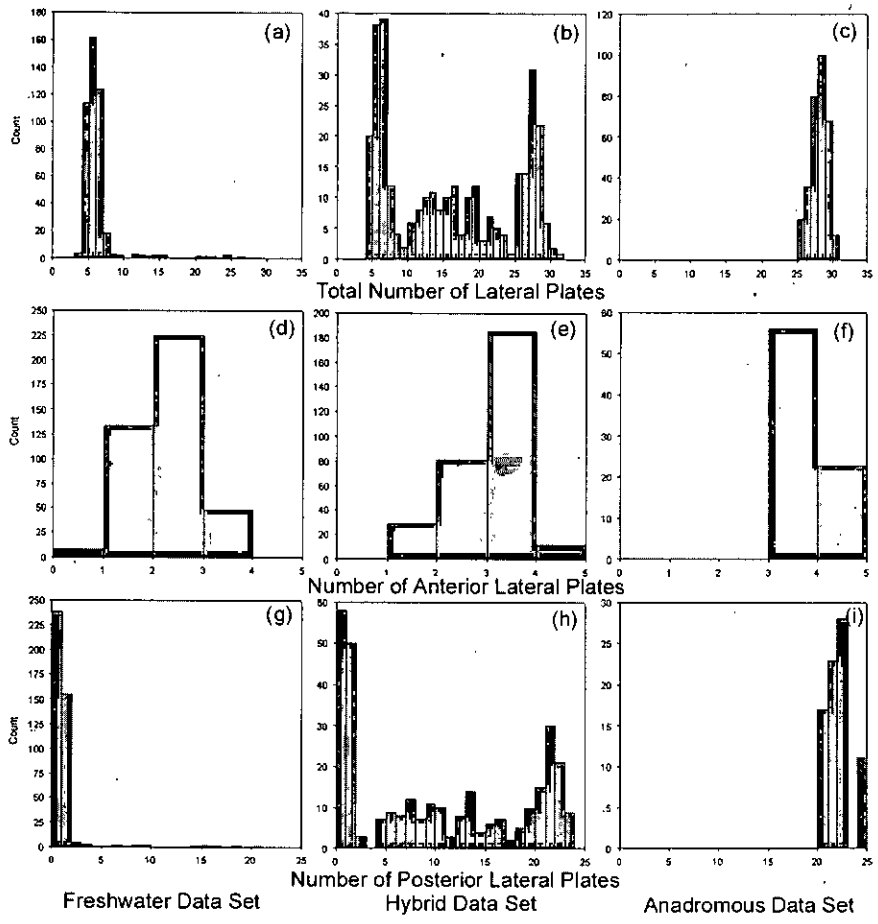


Figure 5.2. Distribution of lateral plate morphology in freshwater, hybrid and anadromous data sets.

Plate Pattern	Fw	Hyb	Anad	Plate Pattern	Fw	Hyb	Anad	Plate Pattern	Fw	Hyb	Anad	Plate Pattern	Fw	Hyb	Anad
0,3,0	2	0	0	0,4,2	0	1	0	3(G2)1(G2)13,3,3	0	1	0	5(LG)4,3,3	0	1	0
0,3,1	101	19	0	1(G1)1,3,3	0	1	0	3(G3)1(LG)10,3,3	0	1	0	5(LG)5,3,3	0	1	0
0,3,2	119	29	0	1(G1)11,3,3	0	1	0	3(LG)1(G2)15,3,3	0	1	0	5(LG)6,3,2	0	1	0
0,3,3	16	9	0	1(G1)5,3,3	0	1	0	3(LG)1,3,3	0	1	0	5(LG)6,3,3	0	1	0
0,3,4	1	0	0	1(G1)6,3,3	0	2	0	3(LG)10,3,2	0	1	0	5(LG)7,3,3	0	1	0
1,2,2	1	0	0	1(G2)3,3,4	0	1	0	3(LG)10,3,3	0	3	0	5(LG)8,3,3	0	3	0
1,3,0	4	1	0	1(G3)5(G1)9,3,3	0	1	0	3(LG)11,3,3	0	1	0	5(LG)9,3,3	0	1	0
1,3,1	33	9	0	1(LG)1(LG)7,3,3	0	1	0	3(LG)4,3,3	0	1	0	5,3,2	0	2	0
1,3,2	102	28	0	1(LG)1,3,3	0	1	0	3(LG)5,2,2	0	1	0	6(G3)12,3,4	0	1	0
1,3,3	15	12	0	1(LG)5,3,3	0	1	0	3(LG)5,3,3	0	2	0	6(LG)14,3,3	0	2	0
2,3,0	1	0	0	10(LG)9,3,3	0	1	0	3(LG)6,3,3	0	2	0	6(LG)7,3,2	0	1	0
2,3,2	1	0	0	10,3,3	0	1	0	3(LG)7,3,3	0	5	0	6(LG)7,3,3	0	1	0
2,3,3	2	1	0	11(G1)8,3,3	0	1	0	3(LG)9,3,3	0	2	0	6,3,2	0	1	0
3(G1)1,3,2	1	0	0	14(G2)4,3,4	0	1	0	4(G1)1(LG)1(LG)9,3,3	0	1	0	7(G1)12,3,3	0	2	0
3(LG)12,3,3	1	1	0	16,3,4	0	1	0	4(LG)1(G1)10,3,3	0	1	0	7(G1)13,3,3	0	2	0
3,2,0	1	0	0	18,3,3	0	1	0	4(LG)12,3,2	0	1	0	7(G3)1,3,3	0	1	0
3,3,2	1	0	0	18,3,4	0	1	0	4(LG)12,3,3	0	2	0	7(LG)2(LG)10,3,3	0	1	0
5,3,3	1	4	0	19,3,3	0	2	0	4(LG)5,3,2	0	1	0	7(LG)9,3,3	0	1	0
6(LG)10,3,3	1	1	0	2(LG)1(G1)9,3,3	0	1	0	4(LG)5,3,3	0	1	0	7,3,2	0	2	0
6(LG)12,3,3	2	0	0	2(LG)1(LG)2,3,3	0	1	0	4(LG)6,3,2	0	1	0	8(G2)11,3,3	0	1	0
6,3,3	2	3	0	2(LG)11,3,2	0	1	0	4(LG)7,3,2	0	1	0	8(G3)10,3,2	0	1	0
7(LG)8,3,3	1	0	0	2(LG)11,3,3	0	1	0	4(LG)8,3,2	0	1	0	8,2,4	0	1	0
7,3,3	1	4	0	2(LG)2(G3)3,3,3	0	1	0	4(LG)8,3,3	0	2	0	8,3,2	0	1	0
8(LG)6,3,3	1	0	0	2(LG)3,3,2	0	1	0	4(LG)9(G2),3,3	0	1	0	9(G1)12,3,3	0	2	0
8,3,3	2	1	0	2(LG)3,3,3	0	1	0	4(LG)9,3,3	0	2	0	9(G3)10,3,3	0	1	0
9,3,3	2	3	0	2(LG)4,3,3	0	1	0	4,3,1	0	1	0	9,3,2	0	1	0
20,3,3	1	7	12	2(LG)5,3,3	0	2	0	4,3,2	0	2	0	**No. of Distinct Plate Patterns**	**27**	**114**	**8**
20,3,4	0	2	5	2(LG)8,3,3	0	2	0	4,3,3	0	2	0				
21,3,3	0	26	16	20,2,4	0	1	0	5(G1)9,3,2	0	1	0				
21,3,4	0	1	7	21,3,2	0	1	0	5(G3)12,3,3	0	1	0				
22,3,3	0	21	19	23,3,3	0	6	0	5(LG)1(LG)14,3,3	0	1	0				
22,3,4	0	0	9	23,3,4	0	2	0	5(LG)1,3,3	0	1	0				
24,3,3	0	0	9	23,3,5	0	1	0	5(LG)10,3,3	0	2	0				
24,3,4	0	0	2	3(G1)12,3,3	0	1	0	5(LG)11,3,3	0	1	0				

Table 5.3. The number of individuals with each lateral plate pattern recorded in each of the three data sets. Fw = freshwater data set, Hyb = Hybrid data set, Anad = Anadromous data set. G1, G2, G3, LG = a gap in lateral plates the size of 1, 2, 3, or >3 (large gap) plates respectively. Commas delimit posterior plates, plates associated with the pelvic girdle, and anterior plates in that order.

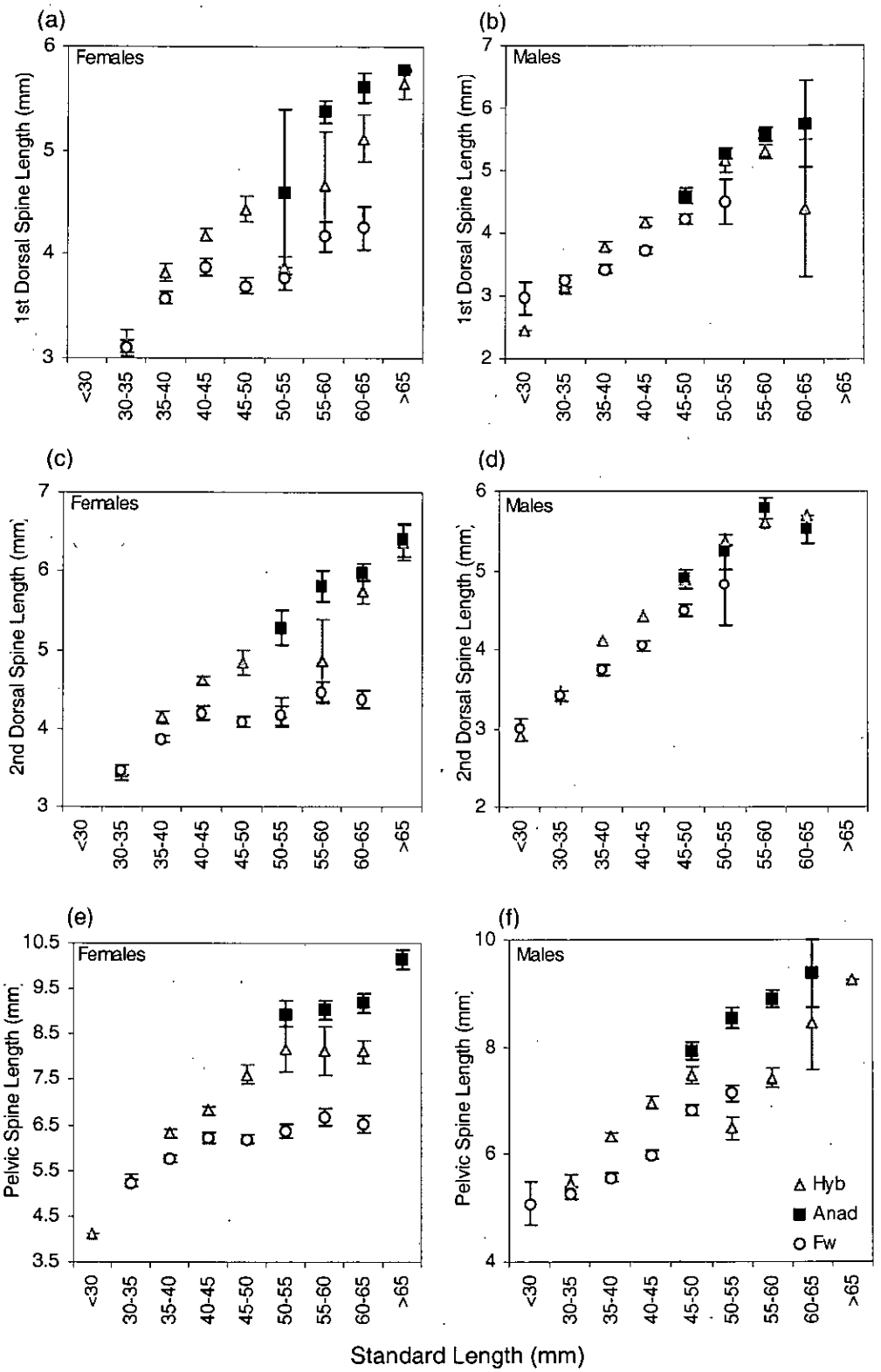


Figure 5.3. Mean 1st dorsal (a,b), 2nd dorsal (c,d) and pelvic spine length (e,f) for each sex and size class based on standard length. Hyb = hybrid data set, Anad = anadromous data set and Fw = freshwater data set.

Tests for Genetic Structure

We found no evidence of population structure within our hybrid data set. The log probability of the data given *K* equal to 1 (-10256.5) was greater than the log probability of the data given *K* equal to 2 or 3 (-10394.3 and -12308 respectively). Analysis of population structure was performed on the anadromous and freshwater data sets elsewhere (Chapter 3), in which we found the most likely value of *K* to be 1 (freshwater data set) and 2 (anadromous data set). We believe that the existence of two clusters in the anadromous data set may be spurious, due to small sample size and highly polymorphic loci (see Appendix Tables 2, 3 and 5), and results in Chapter 3). For the purposes of this study, we assume that the anadromous data set does not have population sub-structure. However, any association between traits and loci found in this data set should be interpreted with this assumption in mind.

Loci with significant differences in allele frequencies between extreme trait groups (R x C χ^2 tests)

Significant differences in allele frequency between extreme trait groups were found in each of the three data sets (Tables 5.4 a, b, and c), and these results are summarised for each data set below.

*Anadromous Data Set (*Table 5.4a)

We observed a significant association between the locus STN208 and 2nd dorsal spine length in anadromous fish. No significant associations were detected between loci and 1st dorsal or pelvic spine length. The loci ATP1a2, STN152 and STN208 were significantly associated with the total number of lateral plates, but these

associations were not observed when testing the number of posterior plates. The significant associations of ATP1a2, and STN152 with total lateral plate number may be largely due to associations with these loci and the number of anterior lateral plates. In addition, the locus STN9 was significantly associated with the number of anterior lateral plates. The loci STN208 and STN96 showed associations with plate pattern groups. After Bonferroni correction for multiple tests, only associations between loci and 2nd dorsal spine length and the number of anterior lateral plates remained significant.

*Hybrid Data Set (*Table 5.*4b)*

Hybrid individuals with long and short 2nd dorsal and pelvic spines differed significantly in allele frequency at the loci STN94 and Cytb. The locus bAR2 was also associated with differences in pelvic spine length in hybrid individuals. Differences in 1st dorsal spine length were associated with significant differences in frequency of alleles at the bAR2 and Cytb loci. We also observed associations between the number of lateral plates (total, posterior, and anterior lateral plates) and the loci Cytb, STN130, and STN208. In addition, differences in the number of anterior plates were associated with differences in frequency of alleles at the STN152 locus. No significant associations were found between loci and the presence or absence of a keel, or between plate pattern. After Bonferroni correction, associations between the loci Cytb and STN94 and 2nd and pelvic spine length remained significant. The association between Cytb and the total number of lateral plates also remained significant.

Freshwater Data Set (Table 5.*4c)*

In freshwater individuals we found significant associations between 2nd dorsal spine length and the loci STN9 and Cytb, and pelvic spine length and the locus STN152. No associations were detected between any of the loci and 1st dorsal spine length. The loci bAR2, STN208 and STN9 were significantly associated with the total number of lateral plates. bAR2 was also associated with the number of posterior lateral plates, and STN9 with the number of anterior lateral plates and plate pattern. In addition, we detected an association between STN96 and the number of posterior plates. The association of bAR2 with total number of lateral plates, and posterior lateral plates remained significant after Bonferroni correction, as did the association between STN96 and posterior lateral plates. Similarly, the association of STN9 with 2nd dorsal spine length, total number of lateral plates and plate pattern was upheld after correcting for multiple tests.

Tables 5.4a-c. P-values of R x C χ^2 tests for associations between loci and traits in anadromous (a), hybrid (b), and freshwater (c) data sets. * p<0.05, ** p<0.01 ***p<0.0001. Values shaded in grey represent significance after sequential Bonferroni correction for 9 (a), or 11 (b) and (c), tests. NA = test not performed due to lack of allelic or trait diversity.

ANADROMOUS STICKLEBACKS								
LOCUS	TRAIT							
	1st Dorsal Spine Length (residual, mm)	2nd Dorsal Spine Length (residual, mm)	Pelvic Spine Length (residual, mm)	No. Lateral Plates (Total)	No. Posterior Lateral Plates	No. Anterior Lateral Plates	Keel (presence / absence)	Plate Pattern
ATP1a2	0.3338	0.0840	*NA*	**0.0323** *	0.6623	**0.0000** ***	*NA*	*NA*
MyoHC	0.9056	0.7936	0.9108	0.7957	0.7941	0.1016	*NA*	0.7143
bAR2	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
CYTb	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
STN130	0.8483	0.6974	0.7719	0.9550	0.9156	0.6108	*NA*	0.5454
STN152	0.5856	0.4260	0.1622	**0.0240** *	0.5570	**0.0044** **	*NA*	0.2281
STN208	0.3274	**0.0047** **	0.7597	**0.0238** *	0.2485	0.2207	*NA*	**0.0103** *
STN26	0.0503	0.0945	0.1271	0.9597	0.8949	0.2473	*NA*	0.8246
STN9	0.5144	0.9643	0.3807	0.0562	0.0825	**0.0015** **	*NA*	0.5785
STN94	0.4790	0.0967	0.7773	0.5955	0.2754	0.4058	*NA*	0.0565
STN96	0.5415	0.2341	0.3301	0.2489	0.0564	0.9728	*NA*	**0.0128** *

Table 5.4b.

HYBRID STICKLEBACKS								
LOCUS	TRAIT							
	1st Dorsal Spine Length (residual, mm)	2nd Dorsal Spine Length (residual, mm)	Pelvic Spine Length (residual, mm)	No. Lateral Plates (Total)	No. Posterior Lateral Plates	No. Anterior Lateral Plates	Keel (presence / absence)	Plate Pattern
ATP1a2	0.7453	0.9202	0.6683	0.1319	0.1326	0.2772	0.0895	0.8157
MyoHC	0.0894	0.4960	0.5500	0.1250	0.0832	0.3898	0.1735	0.1875
bAR2	**0.0251** *	0.1126	**0.0072** **	0.7459	0.6319	0.3393	0.4657	0.8509
CYTb	**0.0201** *	**0.0038** **	**0.0002** ***	**0.0082** **	**0.0119** *	**0.0210** *	0.0804	0.3085
STN130	0.9677	0.6595	0.5722	**0.0192** *	**0.0233** *	**0.0229** *	0.2010	0.6360
STN152	0.2535	0.1844	0.8308	0.2950	0.2872	**0.0082** **	0.4710	0.3197
STN208	0.2658	0.5165	0.8403	**0.0227** *	**0.0053** **	**0.0480** *	0.1162	0.0983
STN26	0.1641	0.2072	0.1942	0.0704	0.0672	0.0873	0.3384	0.1072
STN9	0.9997	0.9874	0.8702	0.6738	0.7216	0.5270	0.7868	0.2823
STN94	0.2109	**0.0006** ***	**0.0012** **	0.6683	0.4692	0.3532	0.3522	0.0569
STN96	0.7791	0.3553	0.7133	0.8777	0.8417	0.9144	0.9530	0.3462

Table 5.4c.

FRESHWATER STICKLEBACKS								
LOCUS	TRAIT							
	1st Dorsal Spine Length (residual, mm)	2nd Dorsal Spine Length (residual, mm)	Pelvic Spine Length (residual, mm)	No. Lateral Plates (Total)	No. Posterior Lateral Plates	No. Anterior Lateral Plates	Keel (presence / absence)	Plate Pattern
ATP1a2	0.1963	0.6476	0.0897	0.8715	0.5407	0.5225	*NA*	0.8174
MyoHC	0.3077	0.8170	0.9855	0.4654	0.9354	0.4742	*NA*	0.1530
bAR2	0.7853	0.4288	0.5358	**0.0003** ***	**0.0000** ***	0.2340	*NA*	**0.0059** **
CYTb	0.0530	**0.0230** *	0.8411	0.7931	0.1896	0.3998	*NA*	0.2278
STN130	0.5312	0.8379	0.4954	0.1939	0.7487	0.1795	*NA*	0.1294
STN152	0.2412	0.3371	**0.0420** *	0.1030	0.7311	0.1652	*NA*	0.2109
STN208	0.6666	0.9335	0.4103	**0.0458** *	0.1775	0.2330	*NA*	0.1428
STN26	0.6605	0.8771	0.4513	0.1527	0.2265	0.4438	*NA*	0.4904
STN9	0.4939	**0.0031** **	0.2982	**0.0030** **	0.4566	**0.0182** *	*NA*	**0.0004** ***
STN94	0.2425	0.5928	0.7925	0.6462	0.1062	0.7718	*NA*	0.1238
STN96	0.5729	0.8196	0.5581	0.0584	**0.0047** **	0.1690	*NA*	0.1056

Associations between alleles and traits (2 x C χ^2 tests)

At loci showing significant associations with a trait, we determined which alleles differed significantly between extreme trait groups in 2 x C χ^2 tests. These alleles are presented in Table 5.5a-c. The loci ATP1a2, Myo3HC, bAR2 and Cytb had only two alleles, the frequency of which could not vary independently. Tests on these loci are essentially the same as those presented in Tables 5.4 a-c and were not repeated, however they are included in Tables 5.5a-c for completeness. As many as four alleles at a given locus differed significantly in allele frequency between extreme trait groups. In hybrid individuals, a similar (but not identical) group of alleles at each of the loci Cytb, STN208 and STN130 differed significantly between groups with few and many total lateral plates, posterior lateral plates and anterior lateral plates. The alleles 185 and 195 (STN94) differed significantly in frequency between individuals with both short and long 2nd dorsal and pelvic spines. In freshwater fish, the alleles 118 and 124 (STN9) differed significantly in frequency between individuals with extreme total number of lateral plates, number of anterior lateral plates and plate pattern. In the anadromous data set, no overlap in alleles associated with different traits were observed apart from alleles at the locus ATP1a2, which differed significantly between individuals with few and many total lateral plates and anterior plates. Many "alleles" that differed significantly between extreme trait groups in anadromous fish were comprised of pooled rare alleles.

Tables 5.5a-c. Summary tables of alleles differing significantly ($p<0.05$) in frequency between extreme trait groups. Non-significant results are not shown. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Number of alleles at loci ATP1a2, MyoHC3, bAR2, and Cytb = 2 for all data sets. The number of alleles at locus STN130 = 6, 7, 3, STN152 = 10, 16, 14, STN208 = 9, 14, 12, STN26 = 6, 6, 2, STN9 = 8, 9, 6, STN94 = 5, 5, 4, STN96 = 7, 7, 6, for anadromous, hybrid and freshwater data sets respectively. p after an allele name represents a pooled class of alleles.

ANADROMOUS STICKLEBACKS

2nd Dorsal Spine Length (residual, mm)					
Locus	*Allele*	*Allele Frequency*		*p*	
		Short	*Long*		
STN208	*125p*	0.215	0.077	**0.046**	*
	133p	0.058	0.327	**<0.001**	***

No. Lateral Plates					
Locus	*Allele*	*Allele Frequency*		*p*	
		Few	*Many*		
Atp1a2	*200*	0.059	0.211	**0.004**	**
STN152	*262p*	0.221	0.050	**0.019**	*
	292p	0.059	0.200	**0.024**	*
STN208	*129p*	0.029	0.250	**<0.001**	***

No. Anterior Lateral Plates					
Locus	*Allele*	*Allele Frequency*		*p*	
		Few	*Many*		
Atp1a2	*200*	0.018	0.381	**<0.0001**	***
STN152	*246p*	0.143	0.000	**0.007**	**
	300p	0.045	0.239	**<0.001**	***
STN9	*112*	0.152	0.000	**0.005**	**
	116	0.089	0.304	**<0.001**	***

Plate Pattern					
Locus	*Allele*	*Allele Frequency*		*p*	
		21,3,3	*22,3,3*		
STN208	*117*	0.342	0.067	**0.004**	**
	123p	0.158	0.400	**0.030**	*
STN96	*223*	0.079	0.267	**0.047**	*
	237	0.263	0.033	**0.006**	**

Table 5.5b.

HYBRID STICKLEBACKS

1st Dorsal Spine Length (residual, mm)					
Locus	*Allele*	*Allele Frequency*		*p*	
		Short	*Long*		
bAR2	*300*	0.177	0.097	**0.025**	*
Cytb	*700*	0.419	0.258	**0.001**	**

2nd Dorsal Spine Length (residual, mm)					
Locus	*Allele*	*Allele Frequency*		*p*	
		Short	*Long*		
Cytb	*700*	0.430	0.247	**<0.001**	***
STN94	*185*	0.077	0.031	**0.050**	*
	195	0.326	0.532	**<0.001**	***
	199	0.125	0.065	**0.048**	*

Pelvic Spine Length (residual, mm)					
Locus	*Allele*	*Allele Frequency*		*p*	
		Short	*Long*		
bAR2	*300*	0.196	0.098	**0.008**	**
Cytb	*700*	0.462	0.280	**<0.001**	***
STN94	*185*	0.016	0.103	**0.000**	***
	195	0.489	0.380	**0.034**	*

No. Lateral Plates					
Locus	*Allele*	*Allele Frequency*		*p*	
		Few	*Many*		
Cytb	*700*	0.444	0.273	**<0.001**	***
STN130	*128*	0.014	0.055	**0.019**	*
	140	0.074	0.032	**0.049**	*
STN208	*119*	0.023	0.064	**0.036**	*
	127	0.171	0.105	**0.045**	*
	131	0.028	0.068	**0.050**	*
	145	0.056	0.005	**0.002**	**

No. Posterior Lateral Plates					
Locus	*Allele*	*Allele Frequency*		*p*	
		Few	*Many*		
Cytb	*700*	0.439	0.275	**0.000**	***
STN130	*128*	0.014	0.055	**0.019**	*
	140	0.074	0.032	**0.050**	*
STN208	*119*	0.023	0.064	**0.036**	*
	131	0.028	0.069	**0.047**	*
	145	0.056	0.000	**0.000**	***

No. Anterior Lateral Plates					
Locus	*Allele*	*Allele Frequency*		*p*	
		Few	*Many*		
Cytb	*700*	0.398	0.282	**0.010**	*
STN130	*128*	0.018	0.059	**0.025**	*
	146	0.018	0.059	**0.025**	*
STN152	*240*	0.159	0.259	**0.010**	**
	244	0.109	0.032	**0.002**	**
	284	0.009	0.051	**0.010**	**
STN208	*119*	0.018	0.064	**0.015**	*
	145	0.068	0.027	**0.043**	*

Table 5.5c.

FRESHWATER STICKLEBACKS

2nd Dorsal Spine Length (residual, mm)						Pelvic Spine Length (residual, mm)					
Locus	*Allele*	*Allele Frequency*		*p*		Locus	*Allele*	*Allele Frequency*		*p*	
		Short	*Long*					*Short*	*Long*		
Cytb	*700*	0.813	0.736	**0.027**	*	STN152	*252*	0.035	0.101	**0.002**	**
STN9	*118*	0.344	0.517	**<0.001**	***		*310*	0.052	0.017	**0.021**	*
	120	0.271	0.184	**0.013**	*						

No. Lateral Plates						No. Posterior Lateral Plates						No. Anterior Lateral Plates					
Locus	*Allele*	*Allele Frequency*		*p*		Locus	*Allele*	*Allele Frequency*		*p*		Locus	*Allele*	*Allele Frequency*		*p*	
		Few	*Many*					*Few*	*Many*					*Few*	*Many*		
bAR2	*300*	0.254	0.396	**<0.001**	***	bAR2	*300*	0.261	0.400	**<0.001**	***	STN9	*118*	0.366	0.458	**0.011**	*
STN208	*171*	0.000	0.034	**0.004**	**	STN96	*237*	0.023	0.078	**<0.000**	***		*124*	0.021	0.049	**0.049**	*
STN9	*118*	0.398	0.485	**0.040**	*												
	124	0.004	0.046	**0.003**	**												

Plate Pattern									
Locus	*Allele*	Allele Frequency						*p*	
		0,3,1	*0,3,2*	*0,3,3*	*1,3,1*	*1,3,2*	*1,3,3*		
bAR2	*300*	0.262	0.409	0.333	0.402	0.256	0.281	**0.005**	**
STN9	*118*	0.381	0.258	0.533	0.500	0.437	0.344	**0.006**	**
	122	0.163	0.212	0.067	0.113	0.113	0.253	**0.049**	*
	124	0.005	0.076	0.000	0.039	0.055	0.156	**<0.001**	***
	132p	0.035	0.015	0.100	0.010	0.046	0.000	**0.045**	*

Linear Models

The minimal models examining associations between traits and the number of allele copies are summarised in Tables 5.6, 5.7, and 5.8 and are discussed for each data set below. Due to the qualitative nature of lateral plate pattern, the significant associations we observed between alleles at the loci STN9 and bAR2 (Table 5.5c) in freshwater sticklebacks could not be explored in a linear model.

Anadromous Data Set (Table 5.6)

We found a significant association between the pooled allele group 129 (locus STN208) and the total number of lateral plates (Table 5.6a, Figure 5.4a). Individuals with one copy of an allele in this pooled group had, on average, one more lateral plate than individuals not possessing a copy of this allele. The presence or absence of this allele explained roughly 11% of the variation in total lateral plate number in the data set. We also found significant associations between the number of anterior lateral plates (3 or 4) and the presence or absence of the alleles at three different loci (allele 100 (ATP1a2), allele 112 (STN9) and the pooled allele group 246 (STN152)). Individuals with allele 100 (ATP1a2) had significantly more anterior lateral plates than those without the allele (Table 5.6b, Figure 5.4b), whilst individuals with the allele 112 (STN9) or one of the pooled alleles 246 (STN152) had significantly fewer anterior lateral plates than those without. Under this model, it is predicted that an individual with the genotype +/-/- with regards to the alleles 100, 112, 246 respectively would be more likely to have 4 anterior plates than 3 (Figure 5.5). The anterior lateral plate model explained 42% of the variation in the data set. Significant associations between the pooled alleles 125 (STN208) and 133 (STN208) observed when comparing individuals with long and short 2nd dorsal spines (Table 5.5a) were not upheld in our linear model analysis of the entire anadromous data set. The presence or absence of either allele did not explain a significant amount of variation in 2nd

dorsal spine length. In anadromous individuals, we also found that individuals with the allele 237 (STN96) were significantly more likely to have the plate pattern 22,3,4 than the plate pattern 22,3,3 (Table 5.6c, Figure 5.4c).

Tables 5.6a-c. Minimal linear models describing variation between alleles and traits in anadromous sticklebacks.

ANADROMOUS STICKLEBACKS

Table 5.*6a*

Total Number of Lateral Plates

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*F ratio*	*df*	*p*
Intercept		27.4394	0.1441			
Allele 129p	STN208	1.0991	0.3552	9.58	1	0.0027 **
Residual mean ss				1.37	77	
Proportion of Variation explained by model:					11.06%	

Table 5.*6b*

Number of Anterior Plates

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*Deviance*	*df*	*p*
Intercept		-1.0647	0.3667			
Allele 100	ATP1a2	2.9365	0.8433	17.49	1	0.0000 ***
Allele 246p	STN152	-8.8874	26.5027	5.64	1	0.0176 *
Allele 112	STN9	-9.7447	24.7085	9.30	1	0.0023 **
Residual deviance				56.19	75	
Proportion of Variation explained by model:					41.04%	

Table 5.*6c*

Plate Pattern

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*Deviance*	*df*	*p*
Intercept		-0.0405	0.4082			
Allele 237	STN96	2.6027	1.1285	8.11	1	0.0044 **
Residual deviance				40.15	33	
Proportion of Variation explained by model:					16.80%	

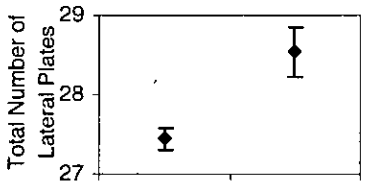


Figure 5.4a

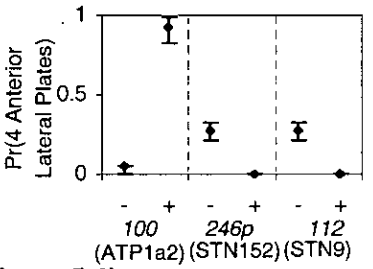


Figure 5.4b

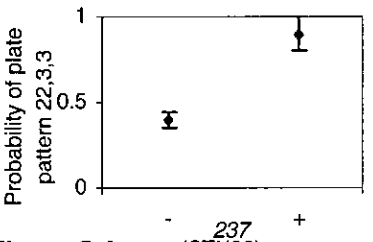


Figure 5.4c

Figures 5.4a-c. Mean trait values (± SE) for anadromous individuals with (+) and without (-) copies of alleles showing significant association with traits in linear models.

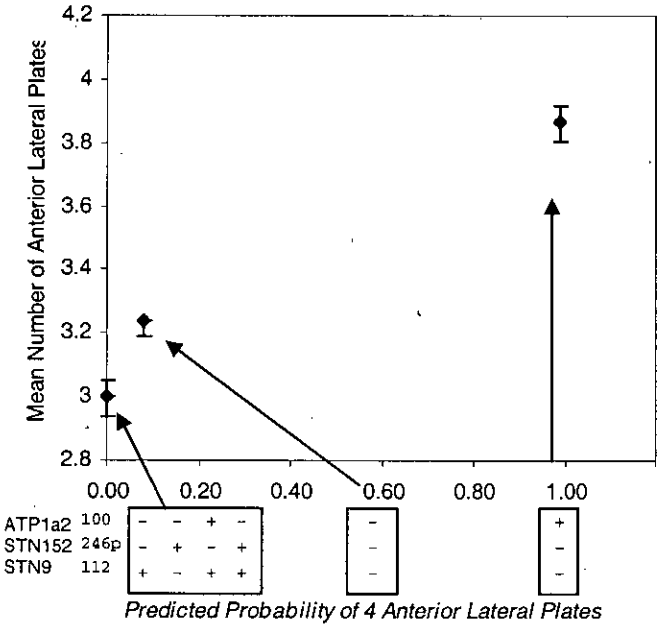


Figure 5.5. Predicted probability of an anadromous individual of given genotype having four lateral plates under binomial linear model plotted against the mean number of anterior lateral plates (±SE) observed for each genotype. Genotypes are symbolised by the presence (+) or absence (-) of a particular allele (specified on the left of the x-axis), and are presented in order of probability from left to right along the x-axis.

Hybrid Data Set (Table 5.7)

We found significant associations between the total number of lateral plates and the alleles 145 (STN208), 128 (STN130), and the allele 800 (Cytb) in hybrid individuals. On average, individuals with a copy of the 145 allele had 6.3 fewer lateral plates than individuals without this allele. In contrast, individuals with the 128 allele and mitochondrial cytochrome b 800 allele had on average 5.7 and 3.6 more lateral plates respectively than individuals without the allele (Table 5.7a, Figure 5.6a). The minimised model predicted that, on average, individuals with the genotype -/+/+ with regards to the alleles 145 (STN208), 128 (STN130) and 800 (Cytb) respectively would have 22.4 lateral plates, whilst individuals with the genotype +/-/- would have 6.8 lateral plates. Figure 5.7a depicts the mean number of lateral plates for each of the sampled genotypes. The minimal model explaining variation in the number of posterior plates is almost identical to that of total number of lateral plates (Table 5.7b, Figures 5.6b, 5.7b). This suggests that no explanatory power is gained by removing variation in anterior lateral plate number. In contrast, the model explaining variation in anterior lateral plate number revealed significant associations with alleles that were different to those associated with posterior and total lateral plate number (except the 800 allele (Cytb), Table 5.7c Figure 5.6c). It should be noted, however, that two of these alleles (119 and 146) were from the same loci as alleles which were associated with posterior and total lateral plate number (STN208 and STN130 respectively). In addition, the allele 244 (STN152) was negatively associated with anterior lateral plate number. From the anterior lateral plate model, it is predicted that on average, individuals with the genotype +/-/-/-, with regards to the alleles 244 (STN152), 119 (STN208), 146 (STN130) and 800 (Cytb) respectively, would have 2.1 anterior lateral plates whilst individuals with the

genotypes -/+/+/+ would have 3.3 anterior lateral plates. The mean anterior plate number for sampled genotypes, and thus the fit of the model, is depicted in Figure 5.7c. Hybrid individuals with the allele 800 (Cytb) had significantly longer 1st dorsal spines than individuals with the alternative 700 allele (Table 5.7d, Figures 6d, 7d). The amount of variation in 1st dorsal spine length explained by the presence and absence of this allele was 2.2%. The presence of the 800 allele was also associated with longer 2nd dorsal spines in contrast to the 195 allele (STN94 locus) which was associated with shorter 2nd dorsal spines (Table 5.7e). Individuals with two copies of the 195 allele had significantly shorter 2nd dorsal spines than individuals with one copy of the 195 allele (Figure 5.6e). The alleles 195 (STN94) and 800 (Cytb) explained 6% of the variation in 2nd dorsal spine length and mean spine length for each genotype is depicted in Figure 5.7e. We detected a positive association between pelvic spine length and the presence of the 185 (STN94) and 300 (bAR2) alleles (Table 5.7f, Figures 6f and 7f). The presence and absence of these alleles explains 5.2% of variation in pelvic spine length.

Tables 5.7a-f. Minimal linear models describing associations between loci and traits in hybrid sticklebacks

HYBRID STICKLEBACKS

Table 5.7a

Total Number of Lateral Plates

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*F ratio*	*df*	*p*
Intercept		13.1140	0.8106			
Allele 145	STN208	-6.3390	2.0923	9.18	1	0.0027 **
Allele 128	STN130	5.7069	2.0351	7.86	1	0.0054 **
Allele 800	Cytb	3.6463	1.0088	13.07	1	0.0004 ***
Residual mean ss				73.55	309	
Proportion of Variation explained by model:					8.93%	

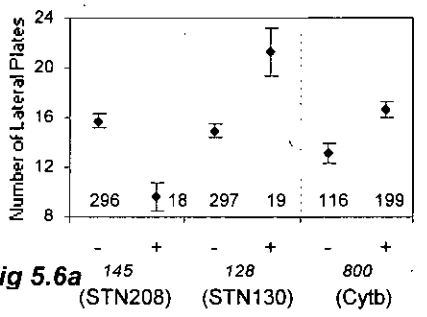


Fig 5.6a

Table 5.7b

Number of Posterior Plates

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*F ratio*	*df*	*p*
Intercept		7.7019	0.7762			
Allele 145	STN208	-6.8913	2.1058	10.71	1	0.0012 **
Allele 128	STN130	5.5596	1.9356	8.25	1	0.0044 **
Allele 800	Cytb	3.3101	0.9699	11.65	1	0.0007 ***
Residual mean ss				66.45	302	
Proportion of Variation explained by model:					9.20%	

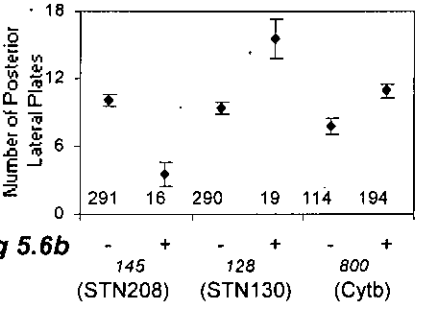


Fig 5.6b

Table 5.7c

Number of Anterior Plates

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*F ratio*	*df*	*p*
Intercept		2.4327	0.0691			
Allele 244	STN152	-0.3465	0.1248	7.70	1	0.0059 **
Allele 119	STN208	0.3532	0.1474	5.74	1	0.0172 *
Allele 146	STN130	0.2867	0.1443	3.95	1	0.0478 *
Allele 800	Cytb	0.2343	0.0840	7.79	1	0.0056 **
Residual mean ss				0.49	297	
Proportion of Variation explained by model:					7.83%	

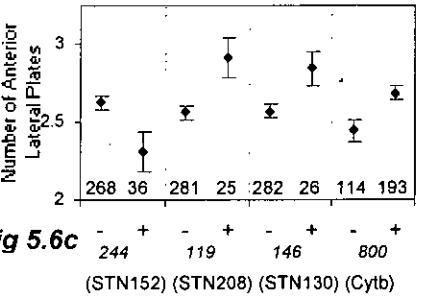


Fig 5.6c

Figures 5.6a-f. Mean trait values (± SE) for hybrid individuals with one(+), two(++), or without (-) copies of alleles showing significant association with traits in linear models. Numbers in plots are sample sizes (N).

Table 5.7 continued.

HYBRID STICKLEBACKS

Table 5.7d

1st Dorsal Spine Length

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*F ratio*	*df*	*p*
Intercept		-0.1097	0.0558			
Allele 800	Cytb	0.1782	0.1248	6.31	1	0.0125 **
Residual mean ss				0.33	276	
Proportion of Variation explained by model:					2.24%	

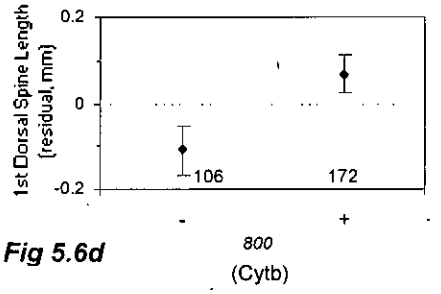


Fig 5.6d

Table 5.7e

2nd Dorsal Spine Length

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*F ratio*	*df*	*p*
Intercept		0.0397				
Allele 195-1copy	STN94	-0.1396	0.0606	6.44	2	0.002 **
-2 copies		-0.2309	0.0666			
Allele 800	Cytb	0.1062		3.89	1	0.049 *
Residual mean ss				0.19		
Proportion of Variation explained by model:					5.96%	

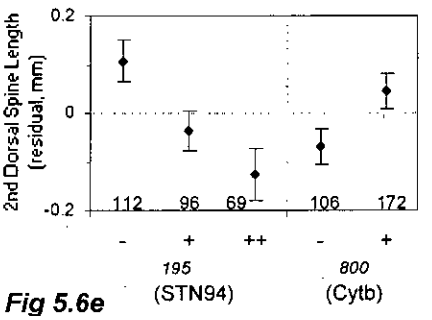


Fig 5.6e

Table 5.7f

Pelvic Spine Length

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*F ratio*	*df*	*p*
Intercept		-0.1563	0.0545			
Allele 185	STN94	0.2555	0.0895	8.15	1	0.0046 **
Allele 300	bAR2	0.1725	0.0614	7.88	1	0.0053 **
Residual mean ss				0.19	272	
Proportion of Variation explained by model:					5.13%	

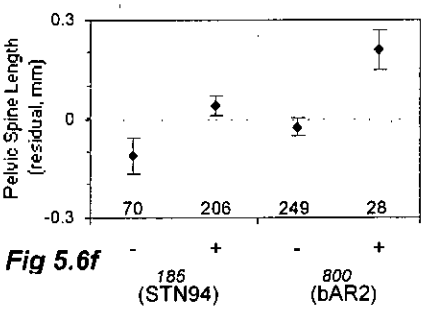


Fig 5.6f

Figure 5.6 continued.

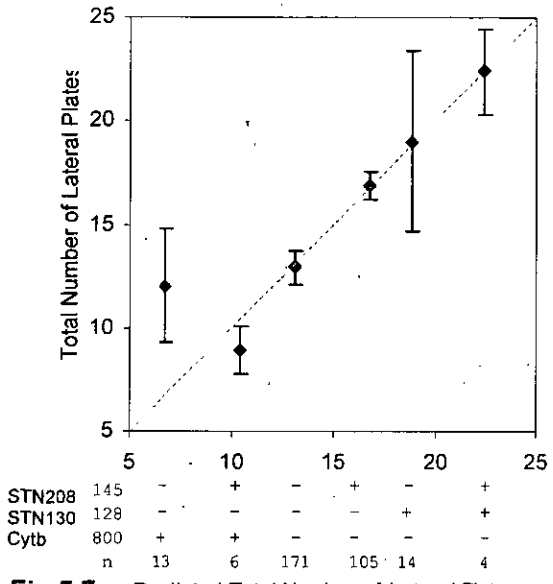


Fig 5.7a Predicted Total Number of Lateral Plates

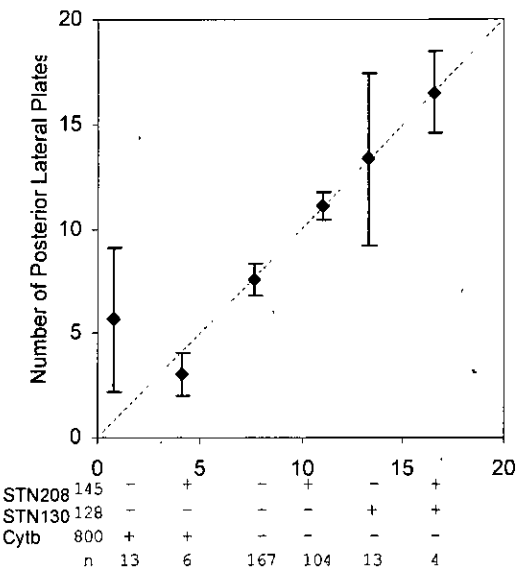


Fig 5.7b Predicted No. of Posterior Lateral Plates

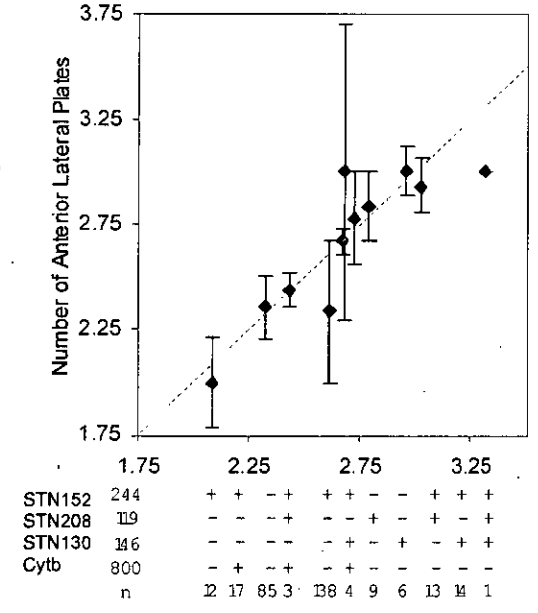


Fig 5.7c Predicted No. of Anterior Lateral Plates

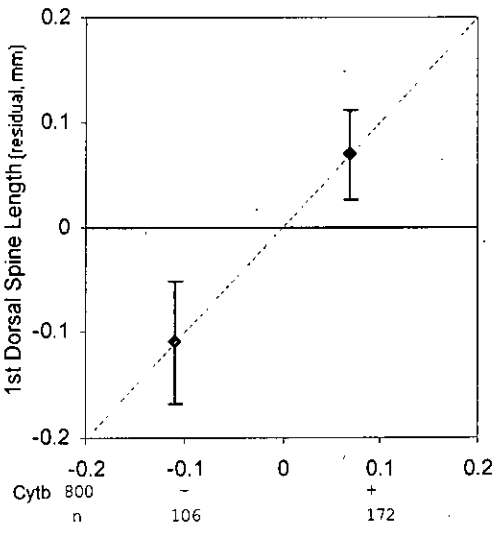


Fig 5.7d Predicted 1st Dorsal Spine Length (mm)

Figures 5.7a-f (continued next page). Predicted trait score of given genotype plotted against the mean trait score (±SE) for that genotype, based on linear models performed on hybrids. Genotypes are symbolised below the x axis by the presence (+) or absence (-) of a particular allele (specified on the left of the x-axis), and are presented in order of predicted score from left to right along the x-axis. N represents sample size.

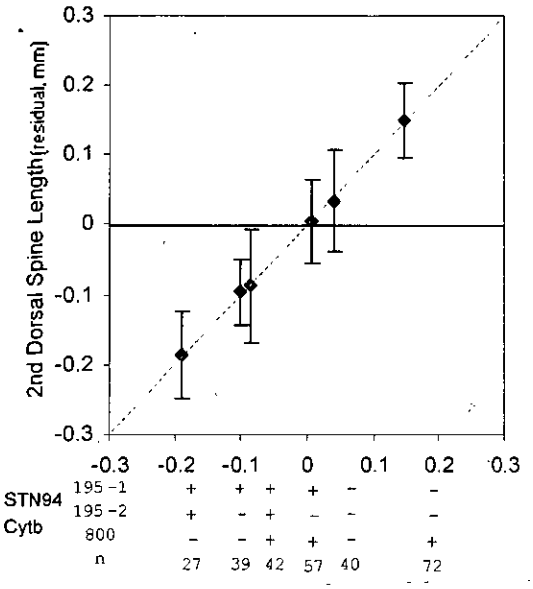


Fig 5.7e Predicted 2nd Dorsal Spine Length (mm)

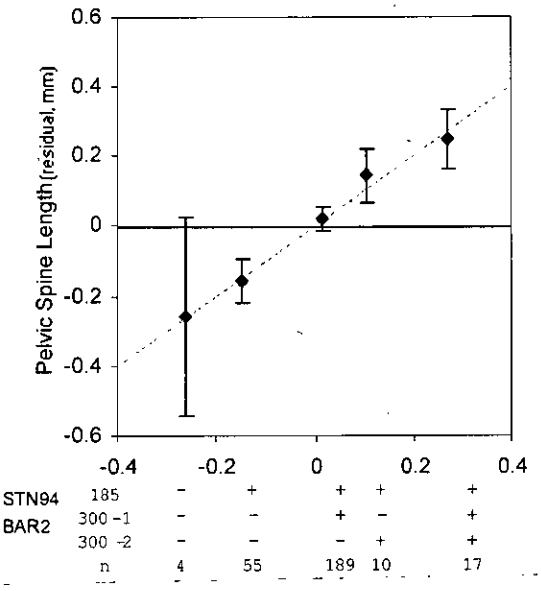


Fig 5.7f Predicted Pelvic Spine Length (mm)

Figure 5.7a-f. continued.

*Freshwater data set (*Table 5.*8)*

We detected a significant negative association between the allele 300 (bAR2 locus) and both the total number of lateral plates and the number of posterior lateral plates (Table 5.8 a and b). Only a small amount of variation explained by the allele 300 was lost by removing variation in the number of anterior lateral plates and examining only variation in the number of posterior lateral plates. Associations between the alleles 118 and 124 (locus STN9) and anterior plate number were not upheld in our linear model analysis of the entire freshwater data set. However, the number of copies of the allele 118 (STN9) explained 18% of the variation in 2nd dorsal spine length in freshwater fish (Table 5.8c). Individuals with two copies of the 118 allele had on average 2nd dorsal spines 0.26mm greater than individuals with no copies of the 118 allele (Figure 5.8c). Variation in pelvic spine length in freshwater fish was significantly explained by two alleles (252 and 310) at the locus STN152 (Table 5.8d). The allele 252 was negatively associated with pelvic spine length whilst the allele 310 was positively associated with pelvic spine length. It is possible that these alleles are not independent (i.e. an increase in the frequency of one allele causes a corresponding decrease in the other allele), however, we believe this is unlikely since we detected more than 14 alleles at the locus STN152 in the freshwater sample.

Tables 5.8a-d. Minimal linear models describing associations between loci and traits in freshwater sticklebacks.

FRESHWATER STICKLEBACKS

Table 5.*8a*

Total Number of Lateral Plates

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*F ratio*	*df*	*p*	
Intercept		6.8846	0.3647				
Allele 300 –1 copy	bAR2	-1.1469	0.4133	9.82	2	0.0000	***
-2 copies		-1.7627	0.4084				
Residual mean ss				6.89	439		
Proportion of Variation explained by model:					4.31%		

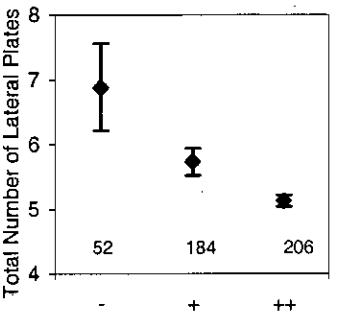


Fig 5.8a *300* (bAR2)

Table 5.*8b*

Number of Posterior Plates

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*F ratio*	*df*	*p*	
Intercept		1.8542	0.3357				
Allele 300 –1 copy	bAR2	-0.8711	0.3785	7.41	2	0.0007	***
-2 copies		-1.3938	0.3760				
Residual mean ss				5.41	411		
Proportion of Variation explained by model:					3.48%		

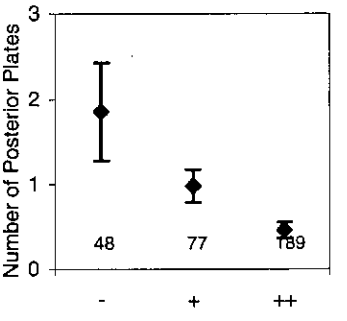


Fig 5.8b *300* (bAR2)

Table 5.*8c*

2nd Dorsal Spine Length

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*F ratio*	*df*	*p*	
Intercept		-0.0721	0.0364				
Allele 118 – 1 copy	STN130	0.0456	0.0468	10.51	2	0.0000	***
- 2 copies		0.2629	0.0597				
Residual mean ss				1.93	429		
Proportion of Variation explained by model:					18.32%		

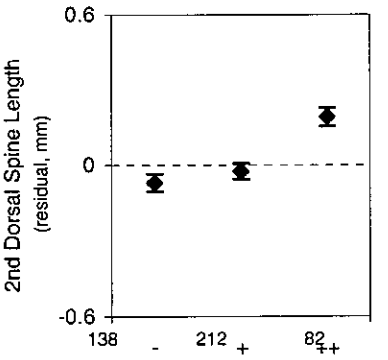


Fig 5.8c *118* (STN130)

Table 5.*8d*

Pelvic Spine Length

Term	*Locus*	*Estimated Coefficient*	*+/- SE*	*F ratio*	*df*	*p*	
Intercept		0.0159	0.0293				
Allele 252	STN152	-0.2618	0.0761	11.83	1	0.0006	***
Allele 310	STN152	0.3111	0.1052	8.75	1	0.0033	**
Residual mean ss				0.30	430		
Proportion of Variation explained by model:					4.68%		

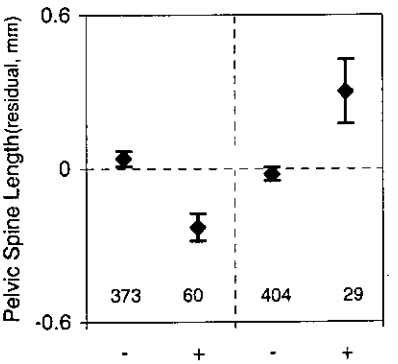


Fig 5.8d *252* *310* (STN152)

Figures 5.8a-d. Mean trait values (± SE) for individuals with one(+), two(++), or without (-) copies of alleles showing significant association with traits in linear models. Figures in plots are sample sizes (N).

DISCUSSION

Our study of associations between traits and loci in Scottish sticklebacks revealed both similarities and differences to QTLs underlying variation in traits in other stickleback populations. The finding of an association between an allele at the locus STN208 and the number of lateral plates (both total plates and posterior plates) in hybrid individuals is consistent with Peichel *et al.* (2001) who reported that alleles at the locus STN208 explained 10% of variation in plate number in Priest Lake sticklebacks (Table 5.1). It is also consistent with Colosimo *et al.* (2004) who reported the marker STN218 (close to STN208 on linkage group XXVI) explained 4% of variation in lateral plate number in their marine-freshwater cross as well as significant variation in their Friant Lake, California, cross. In the present study we found that the 145 allele at the STN208 locus was associated with a difference of 7 fewer lateral plates in sticklebacks from a marine-freshwater hybrid zone in the River Tyne, Scotland. Also in line with another stickleback QTL study (Peichel *et al.* 2001), we found significant associations between the locus STN152 and the number of anterior lateral plates, and the locus STN94 and pelvic spine length in hybrid individuals, and an association between the locus STN130 and 2nd dorsal spine length in freshwater individuals (Table 5.1). The latter association explained a considerable amount of variation (18%) in 2nd dorsal spine length in the freshwater sticklebacks. The significant association we detected between 2nd dorsal spine length and STN94 in hybrids is also of note because STN94 is proximal to the marker Peichel *et al.* (2001) identified as being linked to a 2nd dorsal spine QTL (STN96, linkage group VII). When taken together, these findings provide further support for the notion that the parallel divergence in traits between anadromous and freshwater sticklebacks that has occurred throughout their distribution involves, in part, the same underlying genetic architecture.

In addition to the similarities between the results of our study and the previous studies listed above, we also identified a number of novel associations between loci and traits that differed to those identified in previous studies. For example, the locus STN130 was linked to a QTL explaining variation in 2nd dorsal spine length and gill raker number in Priest Lake sticklebacks (Peichel *et al.* 2001), but in hybrids from the River Tyne we found an allele at STN130 was significantly associated with lateral plate number. These findings suggest that there are differences in the genetic architecture underlying lateral plate variation between Canadian and Scottish sticklebacks. Further, in Scottish sticklebacks multiple QTL could underlie variation in some traits, since in a few cases (e.g. the number of anterior lateral plates in hybrid and anadromous individuals) we found that significant but independent amounts of variation was explained by several alleles at different loci. Flexibility in the genetic determination of traits may have contributed to the rapid adaptive radiation in sticklebacks that has occurred in the last 20,000 years by allowing selection pressures in novel environments to favour advantageous mutations at a number of different locations throughout the genome.

A noticeable trend in our association analysis of hybrid individuals, was the association between alleles at the mitochondrial cytochrome b locus and several different traits (lateral plate number, 1st and 2nd dorsal spine length). It is generally thought that mtDNA is maternally inherited (but see Zouros *et al.* 1992, Kondo *et al.* 1992 and Bromham *et al.* 2003), and as a result, mutations accumulate along maternal lines. Mutations in the mitochondrial genome are known to have an effect on fitness (e.g. Martin and Leob 2004, Wallace 2001) but fitness benefits may depend on the nuclear genetic background (Hutter and Rand 1995). It is highly unlikely that the associations we observed between mtDNA alleles and several different traits are a result of a QTL in the mitochondrial genome. Rather, we argue

that these consistent associations with several different traits are more likely to stem from the existence of a high proportion of F1 hybrids in our hybrid sample resulting in strong cytonuclear disequilibrium. The diagnostic nature of the cytochrome b allele between anadromous and freshwater sticklebacks in this system means that associations between the mitochondrial alleles and anadromous and freshwater traits would be strong in F1 hybrids. Associations might also exist between traits and other nuclear loci.

We found only weak evidence of consistent associations in anadromous, freshwater and hybrid sticklebacks. The loci STN208 and STN152 showed significant associations with the total number of lateral plates and the number of anterior lateral plates in both anadromous and hybrid individuals. However, the precise alleles showing associations at these loci differed, with significant differences in lateral plate number being associated with pooled rare alleles in anadromous individuals. Additionally, the direction of association of the particular alleles at the locus STN208 differed (a positive association in anadromous individuals and a negative association in hybrid individuals). The lack of consistent associations in all three data sets is not surprising because trait variation differed between anadromous, hybrid and freshwater individuals and, therefore, we had no reason to expect that we would detect the same associations between marker loci and traits. For example, a locus associated with lateral plate number in hybrid individuals might be fixed for low or high plated alleles in freshwater and anadromous individuals respectively. If this is the case, then associations we observe in freshwater and anadromous sticklebacks may describe variation explained by QTL of minor effect. Thus, although in freshwater sticklebacks an allele at the locus STN130 explained 18% of variation in 2nd dorsal spine length, this marker may be linked to a QTL of minor effect in terms of differences in spine length between anadromous and freshwater sticklebacks. In freshwater sticklebacks, we also found significant associations between alleles at the

locus bAR2 and the number of lateral plates (total number of plates and posterior plates) and, at present, the position of this marker on the stickleback linkage map is unknown. In addition, two different alleles at the locus STN152 were found to be associated with pelvic spine length in freshwater individuals. The independent effects of these alleles are interesting because STN152 was associated with lateral plate number, not pelvic spine length, in Priest Lake sticklebacks. This provides further evidence to suggest that differences in QTL exist between Priest Lake and River Tyne sticklebacks.

This study highlights some of the difficulties encountered when performing association analyses on a wild population, particularly in hybrid zones. One problem general to association studies is that the associations we detect may be "spurious", since the large number of tests performed makes association studies prone to increased type I error rates. In addition, the relatively small sample sizes available from wild populations combined with high allelic diversity means that the power to detect associations is low, even if pedigree data are available. Further, environmental factors (e.g. microhabitat variation) in wild populations will lead to increased trait variance (e.g. wear and tear on dorsal spines) compared to laboratory populations bred under constant conditions, thus reducing the power to detect QTL even further. Studying marker-trait associations in hybrid zones introduces additional problems of linkage disequilibrium and epistasis. Although the phenotypic diversity within hybrid zones makes them useful for studies of QTL associated with species divergence, one of the major difficulties of our approach is distinguishing between associations due to physical linkage and associations due to linkage disequilibrium and epistasis. Rieseberg and Buerkle (2002) suggest that careful sampling strategies avoiding early generation hybrids (e.g. 1^{st} –4^{th} generation) might circumvent these difficulties, but in a hybrid tension zone maintained by a balance between selection and migration, this may be difficult to achieve. Nevertheless, the

fact that we observed associations between traits and loci that were consistent with other stickleback populations, despite using a sample of wild sticklebacks of unknown pedigree, is interesting and suggests that this approach is of use when studying wild populations and worthy of further investigation. Determining pedigree structure in wild hybrids may limit spurious associations due to linkage disequilibrium but would not remove the problem of epistatic effects on a QTL. A further problem is that the effect of a QTL on a trait might depend on the environmental background (Verhoeven *et al.* 2004), as well as being influenced by the nuclear genetic background (Mackay 2004).

Despite all of the above mentioned difficulties, the large phenotypic differences between two source populations and presence of recombinant genotypes within a hybrid zone provides a good opportunity to study QTL associated with divergence and speciation in wild populations. In this paper, we have addressed some of the difficulties of this approach by targeting specific candidate loci and alleles, and looking for associations in hybrids as well as in source populations. We found significant associations between traits and loci, some of which were similar and some of which were different to those found in other stickleback populations. These results require verification either by constructing pedigrees from wild sticklebacks or performing laboratory crosses. Nevertheless, our findings suggest both stability and flexibility in the genetic architecture underlying morphological divergence and parallel evolution in sticklebacks. It is possible that this flexibility may be the underlying reason for the rapid, multiple and independent adaptation of anadromous sticklebacks to freshwater habitats.

CHAPTER SIX

DISCUSSION

In this thesis I have highlighted how anadromous and freshwater sticklebacks offer an excellent opportunity to study the evolution of reproductive isolation. The parallel divergence of morphotypes and the existence of these forms in sympatry in estuaries throughout the Northern hemisphere makes them an ideal model system to investigate questions related to speciation. Although a great deal is already known about the evolutionary biology of the threespined stickleback (e.g. Bell and Foster 1994), evidence of both premating and postmating isolation in wild populations was lacking. Indirect evidence suggests that both occur and that ecological factors may play a significant role in maintaining divergence between the morphs (McKinnon and Rundle 2002). This interplay with ecological factors makes studying wild populations particularly important. The primary aim of this thesis was to examine premating and postmating isolation of marine and freshwater sticklebacks in the wild (the River Tyne, East Lothian, Scotland). Here, I summarise the findings and discuss what implications these have for our understanding of divergence between anadromous and freshwater sticklebacks as a whole.

Basic biology and life history of sticklebacks in the River Tyne

Freshwater sticklebacks in the River Tyne have a one year life span with limited overlap between generations (Chapter 2). My observations showed that in the River Tyne the earliest fry hatch in late June, and by September population sizes of sticklebacks peak (F. Jones, unpublished data). Sticklebacks show very high site philopatry at all times of the year (F. Jones, unpublished data), although the extent of dispersal of individuals less than 25mm is unknown (and may occur during floods). Migration of juvenile anadromous sticklebacks out to sea appears to occur

primarily between September and October (Chapter 2). Adult anadromous sticklebacks migrate from the ocean into the rockpools and lower reaches of the River Tyne between late April and early May to breed. The breeding season of resident-freshwater sticklebacks starts slightly earlier, with gravid females being sampled in early April. Freshwater sticklebacks die in July-August shortly after breeding. It is unknown whether anadromous sticklebacks die or migrate back out to sea. Although for freshwater sticklebacks a one-year lifespan is common, populations of both freshwater and anadromous sticklebacks have been reported to breed for more than one year elsewhere (Bell and Foster 1994).

Facultative anadromy is likely to have played a significant role in the adaptive radiation of sticklebacks into freshwater environments throughout their distribution. The ability of anadromous sticklebacks to migrate upstream will be impeded by both natural (e.g. stream gradient) and man-made barriers (e.g. weirs). We found evidence to suggest that the weir upstream of site 4 in the River Tyne imposed a significant barrier to upstream migration (Chapter 3) and this is likely to promote divergence between anadromous and freshwater sticklebacks. The extent of downstream geneflow remains to be investigated.

Evidence of morphological and genetic divergence

Anadromous and freshwater sticklebacks in the River Tyne differ significantly in a number of morphological traits, the most obvious being body size (Chapter 2). The larger size of anadromous fish is due to their migration into food-rich marine habitats (Bell and Foster 1994) but it is also likely to have a genetic basis (Colosimo *et al.* 2004). Anadromous and freshwater sticklebacks in the River Tyne also differ

significantly in body shape, plate number (Chapter 2), and spine lengths, however, comparisons of the spine lengths and other growth-affected traits are difficult to make across the hybrid zone due to site-specific differences in allometry (Chapter 3). The morphological differences we observed between anadromous and freshwater sticklebacks in the River Tyne are consistent with differences reported in many other stickleback populations (Hagen 1967, McPhail 1994, Walker and Bell 2000), but are not as extreme as those observed between isolated populations of anadromous and freshwater sticklebacks in other parts of Scotland (F. Jones, Pers. Obs.). The relatively small morphological differentiation between sticklebacks in the River Tyne is most likely due to continuing low levels of geneflow between anadromous and freshwater morphs. We found significant differences in allele frequencies between anadromous and freshwater sticklebacks at microsatellite, intron and mitochondrial markers (Chapters 3, and 4, and unpublished data), indicating the existence of distinct gene pools.

Evidence for reproductive isolation

In sites 2-4 of the River Tyne, anadromous sticklebacks bred in spatial and temporal sympatry with resident freshwater sticklebacks (Chapter 2). The frequency of intermediate morphotypes and individuals of intermediate genetic ancestry during this time was relatively low. Within a site, both anadromous and freshwater morphs were caught in a given trap indicating that microhabitat differences (if they exist at all) are not strong. A further field based study should verify this and provide further details regarding the exact location of nesting sites. Based on their size-manipulative mate choice experiment, McKinnon *et al.* (2004) argue that size-assortative mating (sexual selection) is an important premating barrier between

anadromous and freshwater sticklebacks. Size-assortative mating appears to be a common theme in premating isolation of other anadromous and freshwater species pairs and has been implicated to play a role in premating isolation between char (*Salvelinus malma* and *S. confluentus,* Maekawa *et al.* 1993) and salmon (sockeye and kokanee; *Oncorhynchus nerka,* Foote and Larkin 1988). However, in sympatric sticklebacks from the River Tyne, we observed only a weak preference for larger males and size-assortative mating did not appear to be important in preventing hybridisation in the experimental ponds (Chapter 2). The semi-natural conditions in the experimental ponds may differ from conditions in the wild (e.g. lack of water flow, nesting density, microhabitat structure). On the basis that we found evidence of reproductive isolation in the wild (Chapter 3) but not in the experimental pond manipulation (Chapter 2), it seems likely that the environment either mediates assortative mating between anadromous and freshwater sticklebacks in this river, or drives selection against hybrid fry. The exact nature of this interaction remains to be determined, however, some clues may be gained by examining other anadromous-freshwater species pairs. For example, similar interactions between the environment and mate choice may also exist in sockeye and kokanee salmon. Sockeye (anadromous) and kokanee (resident freshwater) salmon have a similar evolutionary history to that of sticklebacks with parallel divergence in morphology and genetics throughout their range since the last glacial maximum (Taylor 1999). There is some evidence to suggest that sockeye and kokanee mate assortatively (Foote and Larkin 1988) and that this mate choice is primarily based on size. Like sticklebacks, the form adopting an anadromous life history strategy (sockeye) has a larger body size at maturity due to the greater productivity of the marine environment (although body size is also likely to have a genetic component, *Hankin*

et al. 1993). When spawning, this size advantage enables sockeye males to out-compete kokanee males for the best spawning positions and greater body size makes sockeye females more desirable as mates than kokanee females. In direct contrast, McLean *et al.* (2004) found no evidence of size assortative mating in wild sockeye salmon, but here genetic markers (as apposed to observational data) were used to assign parentage. This discrepancy may be explained by differences in experimental approach or environmental variation between rivers. Wood and Foote (1996) found evidence of differences in spawning microhabitat preference between sockeye and kokanee salmon (e.g. differences in water velocity, gravel size, and redd depth at nesting sites). Variation in spawning microhabitat choice may be mediated via competitive exclusion between the morphs. Therefore, population density, as well as the number and microhabitat of spawning locations within the environment may influence the degree of size-assortative mating that occurs within any one sympatric population. In this way, I argue that the environment plays an important role in mediating reproductive isolation between anadromous and freshwater fish.

Evidence for postmating isolation

Although we conclude that ecology-dependent reproductive isolation exists between anadromous and freshwater sticklebacks in the River Tyne, this conclusion is based on an observed heterozygote deficit, and cytonuclear disequilibrium in juveniles. An alternative explanation for these observations would be the occurrence of either endogenous or exogenous selection against fry in the wild. Although we did not test for endogenous selection against hybrid fry directly, we do not believe that genetic

incompatibilities are manifested in River Tyne hybrid fry because we did not observe a deficit of fry from matings between anadromous and freshwater morphotypes in our pond experiment (Chapter 2). In contrast, we are unable to rule out the occurrence of exogenous selection against hybrid fry in the wild River Tyne population. This remains to be tested by comparing allele frequencies in fry and juvenile samples from the same cohort.

The relatively high frequency of juvenile hybrid sticklebacks observed in the River Tyne indicates that premating isolation is not complete. In the absence of selection against recombinant individuals, the occurrence of geneflow would have the effect of breaking down divergence between anadromous and freshwater sticklebacks. Therefore, for the observed differences between anadromous and freshwater sticklebacks in the River Tyne to be maintained, there must be some degree of postmating isolation either within or without the hybrid zone. In chapter four, we outlined several lines of evidence for selection against recombinant individuals (both genetic hybrids and morphological intermediates) in the River Tyne. These included a steeper cline in body size than in lateral plate number and body shape which suggests that body size may be of particular importance for adaptation to alternative marine and freshwater environments. In salmonids, body size plays an important role in the determination of a migration tactic and most evidence suggests that a size threshold must be reached before migration is adopted (e.g. Theriault and Dodson 2003, Okland *et al.* 1993). Smaller and slower growing fish delay migration to the following year whilst large fish either migrate or stay resident (Theriault and Dodson 2003). Standard length in sticklebacks has a heritable component (Colosimo *et al.* 2004) and divergence in this trait may reflect trade-offs in growth

investment in the resource limited freshwater environment. Divergence in body size is also common in other fish species which show facultative anadromy (e.g. salmon, Foote and Larkin 1988, trout, Jonsson *et al.* 2001, charr, Theriault and Dodson 2003).

Other evidence for selection in the River Tyne system includes the reduced probability of overwinter survival of genetic hybrids (Chapter 4). This is most likely to be a result of exogenous selection against hybrids since genetic incompatibilities have not been observed in hybrid anadromous-freshwater sticklebacks (McPhail 1994). Our finding contrasts directly with Hagen's (1967) study of anadromous-freshwater stickleback hybrids in the Little Campbell River and this difference may stem from differences in experimental approach or from differences in selection pressures acting in the two rivers. Reduced overwinter survival may be a result of inability to survive exposure to extreme winter climatic conditions and this may stem from decreased energy reserves. For example, Hutchings *et al.* (1999) found that overwinter survival probability in brook trout *(Salvelinus fontinalis)* was correlated with the degree of lipid reduction. Climatic conditions over winter are more likely to affect the survival of hybrids inhabiting freshwater riverine environments because these environments are less buffered against climatic extremes than the ocean. However, reduced overwinter survival may also stem from a reduced ability to survive an anadromous migration, and this might be caused by multiple different factors (e.g. poor body condition, foraging or homing ability, and increased susceptibility to predation). Hybrids adopting an anadromous life history strategy might be more prone to physiological distress due to changes in water chemistry associated with their life history. Both selection acting on hybrid individuals within

and outside the hybrid zone in the River Tyne is likely to be occurring (Chapter 4), and the migratory behaviour of hybrids should be investigated further. Analysis of juvenile body condition prior to the onset of winter may also provide clues as to the reason for the reduced overwinter survival of hybrids we observed. Aside from reduced overwinter survival of genetic hybrids, we did not detect any other strong evidence of reduced fitness. Female genetic hybrids surviving to the breeding season have equal probability of being gravid in the breeding season, and juvenile genetic hybrids were of intermediate body size, compared to anadromous and freshwater individuals. In this analysis, body size was regarded as an indirect measure of fitness because it is correlated with reproductive success, however, it should be noted that intermediacy in body size, or other traits, may render a hybrid individual less fit in either freshwater or marine environments.

Independent of genetic ancestry, we found further evidence of selection against a specific trait; lateral plate number (Chapter 4). Lateral plate morphology is one of the most studied traits in the threespined stickleback, yet the factors influencing its repeated divergence in stickleback populations are poorly understood. To my knowledge we are the first to find evidence for selection acting on intermediate lateral plate morphology in a wild stickleback population. Females of intermediate plate number in the River Tyne were of smaller size in the juvenile stage, showed reduced probability of overwinter survival and reduced probability of being gravid. It is likely that reduced juvenile body size in females of intermediate plate number has knock-on effects on overwinter survival and adult reproductive ability. The finding of reduced fitness in females but not in males, is particularly interesting because it suggests sex-biased selection. Whilst a purely genetic basis for the observed

reduced fitness cannot be ruled out (e.g. Rand *et al.* 2001 suggest sex biased selection is necessary to maintain functional cytonuclear epistasis between genes), I propose that reduced fitness in females is likely to have an ecological basis (such as sex differences in foraging behaviour or parasite load) and this is currently being investigated.

This study provides the first direct evidence of selection against hybrids and intermediate morphotypes in the wild. Previous laboratory experiments (Hagen 1967, McPhail 1994) and observations of a wild population (Hagen 1967) failed to find hybrid inviability. This contrasts with our results and highlights the importance of ecology-dependent selection as a postmating barrier to geneflow. The influence of environment on the occurrence of hybridisation is a recurring theme in fish speciation but the relative importance of premating and postmating barriers to reproductive isolation appear to differ with both the environments and species involved. Conclusions from our study of postmating isolation between anadromous and freshwater sticklebacks are similar to those drawn from a study of coastal cutthroat and coastal steelhead trout (Ostberg *et al.* 2004), but in direct contrast to conclusions drawn from studies of postmating isolation in cutthroat and rainbow trout (e.g. Leary *et al.* 1995). In laboratory conditions, Leary and colleagues (1995) found reduced hybrid survival of hybrid cutthroat and rainbow trout. Spatial and temporal differences in spawning behaviour are thought to play a major role in premating isolation between coastal cutthroat and rainbow trout (*Oncorhynchus clarki and O. mykiss,* Hitt *et al.* 2003) and differences in environmental conditions influences the occurrence of hybridisation (Campton and Utter 1987, Young *et al.* 2001). Where hybridisation does occur, postmating isolation may preserve species

integrity since backcrosses are less frequent than expected under an assumption of equal fitness (Young *et al.* 2001). However, in a detailed study of wild stream-resident populations of trout no evidence of hybrid inferiority was observed (Rubidge and Taylor 2004).

Associations between traits and markers

In Chapter five, we investigated associations between candidate marker loci and traits and found that some loci associated with traits in a Canadian lake population were also associated with traits in sticklebacks from the River Tyne. In common with previous studies (Peichel *et al.* 2001, Colosimo *et al.* 2004), we found the loci STN208 and STN152 to be associated with lateral plate number, and STN94 to be associated with pelvic spine length in hybrids. We also found the locus STN130 to be associated with 2nd dorsal spine length in freshwater sticklebacks from the River Tyne. These findings suggest that, in part, the same underlying genetic architecture is involved in the parallel divergence in traits between anadromous and freshwater sticklebacks in different parts of their distribution. However, the finding of statistical associations between markers and traits that were not observed in other stickleback populations, is indicative of flexibility in the underlying genetic architecture associated with trait divergence/evolution in sticklebacks. This analysis highlighted some of the major drawbacks of studying marker associations in wild populations and hybrid zones in particular. Spurious associations are likely to exist due to the large number of tests performed, as well as the presence of linkage disequilibrium or epistatic selection (selection for particular gene combinations) in the hybrid sample. Determining family structure in samples from the wild, and applying a transmission-

disequilibrium test may provide a more powerful approach for detecting true associations (i.e. associations due to physical linkage) between traits and markers.

Conclusions

My study of sticklebacks in the River Tyne, Scotland has shown empirical evidence of both reproductive isolation in the form of postmating barriers to geneflow between anadromous and freshwater sticklebacks. The finding of reproductive isolation in wild populations of anadromous and freshwater sticklebacks is consistent with laboratory based studies of assortative mating (e.g. Hagen 1967, Ziuganov 1995, Scott 2004, McKinnon *et al.* 2004), but is unique in showing direct evidence for postmating isolation in a wild population (compare to Hagen 1967). Premating barriers to geneflow between River Tyne sticklebacks may exist and could involve ecology-dependent assortative mating. Additional postmating barriers to geneflow include selection against individuals of hybrid genetic ancestry, as well as sex-biased selection against intermediate lateral plate morphotypes. From my findings it is apparent that ecology-dependent selection plays a large role in maintaining or driving divergence between anadromous and freshwater sticklebacks.

Future directions

As the first detailed genetic study of a stickleback hybrid zone, this work lays the foundation for many future studies and highlights the utility of *in situ* studies of wild stickleback populations. Throughout this thesis, I have outlined areas requiring further investigation which would supplement our understanding of stickleback evolution. The existence of microhabitat variation in nesting sites, early hybrid fry mortality, and ecology-dependent courtship behaviour should be investigated in the

wild. The migratory behaviour of hybrids and whether the reduced overwinter survival of genetic hybrids is due to limited energy reserves are areas also worthy of investigation. Whether developmental instability, increased parasite load or diminished foraging efficiency causes reduced juvenile size of females with intermediate plate morphology is already being investigated and will provide insight into the immediate factors maintaining divergence in lateral plate morphology. The existence of family structure and relatedness of individuals within the River Tyne would also be an interesting area of further investigation and the presence of sibships may provide a more statistically robust method of detecting associations between marker loci and traits. Since ecology-dependent selection is important in maintaining divergence between anadromous and freshwater sticklebacks in this river system, a comparison of other hybrid zones in a number of rivers using the same genetic approach would enable the generality of factors affecting divergence between sticklebacks to be assessed.

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APPENDIX

SUMMARY GENETIC DATA

Table 1. Sample sizes of individuals genotyped from each site, each month. Age Class A = Adult, Age Class J = Juvenile.

Year		2001	2002			2003													Grand
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Site
Age class		A	A	J	J	A					A	J						J	Total
Site	1	19	-	-	-	-	34	20	16	1	**71**	-	-	-	-	-	-	**0**	**90**
	2	-	-	-	45	14	38	50	1	-	**103**	39	50	27	5	3	6	**130**	**278**
	3	-	-	-	45	23	42	50	5	-	**120**	32	50	50	30	13	15	**190**	**355**
	4	75	18	40	50	50	45	35	7	-	**137**	22	50	50	30	30	23	**205**	**525**
	5	-	-	-	45	-	-	-	-	-	**0**	-	-	50	-	-	-	**50**	**95**
	6	-	-	-	45	-	-	-	-	-	**0**	-	-	50	-	-	-	**50**	**95**
	7	51	21	6	45	30	36	50	39	-	**155**	11	50	44	30	30	30	**195**	**473**
	8	-	-	-	-	-	-	-	-	-	**0**	-	50	-	-	-	-	**50**	**50**
Total		**145**	**39**	**46**	**275**	**117**	**195**	**205**	**68**	**1**	**586**	**104**	**250**	**271**	**95**	**76**	**74**	**870**	**1961**

Table 2. The number of alleles sampled at each locus from each site each month. Totals represent the number of distinct alleles sampled.

ATP1a2

Year		2001	2002			2003													Overall
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Total
Age class		A	A	J	J	A					A	J						J	
Site	1	2	-	-	-	-	2	2	1	n/a	2	-	-	-	-	-	-	-	2
	2	-	-	-	2	2	2	2	n/a	-	2	2	2	2	1	2	2	2	2
	3	-	-	-	2	2	2	2	2	-	2	2	2	2	2	2	2	2	2
	4	2	2	2	2	2	2	2	2	-	2	2	2	2	2	2	2	2	2
	5	-	-	-	2	-	-	-	-	-	-	-	-	2	-	-	-	-	2
	6	-	-	-	2	-	-	-	-	-	-	-	-	2	-	-	-	-	2
	7	2	2	1	2	2	2	2	2	-	2	1	2	2	2	2	2	2	2
	8	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1
																	Grand Total		2

MyoHC

Year		2001	2002			2003													Overall
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Total
Age class		A	A	J	J	A					A	J						J	
Site	1	2	-	-	-	-	2	2	2	n/a	2	-	-	-	-	-	-	-	2
	2	-	-	-	2	2	2	2	n/a	-	2	2	2	2	2	2	2	2	2
	3	-	-	-	2	2	2	2	2	-	2	2	2	2	2	2	2	2	2
	4	2	2	2	2	2	2	2	2	-	2	2	2	2	2	2	2	2	2
	5	-	-	-	2	-	-	-	-	-	-	-	-	2	-	-	-	-	2
	6	-	-	-	2	-	-	-	-	-	-	-	-	2	-	-	-	-	2
	7	2	2	2	2	2	2	2	2	-	2	2	2	2	2	2	2	2	2
	8	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	2
																	Grand Total		2

bAR2

Year		2001	2002			2003													Overall
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Total
Age class		A	A	J	J	A					A	J						J	
Site	1	1	-	-	-	-	1	1	1	n/a	1	-	-	-	-	-	-	-	1
	2	-	-	-	2	2	2	2	n/a	-	2	2	2	2	2	2	2	2	2
	3	-	-	-	2	2	2	2	2	-	2	2	2	2	2	2	2	2	2
	4	2	2	2	2	2	2	2	2	-	2	2	2	2	2	2	2	2	2
	5	-	-	-	2	-	-	-	-	-	-	-	-	2	-	-	-	-	2
	6	-	-	-	2	-	-	-	-	-	-	-	-	2	-	-	-	-	2
	7	2	2	2	2	2	2	2	2	-	2	2	2	2	2	2	2	2	2
	8	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	2
																	Grand Total		2

Cytb

Year		2001	2002			2003													Overall
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Total
Age class		A	A	J	J	A					A	J						J	
Site	1	1	-	-	-	-	2	2	1	n/a	**2**	-	-	-	-	-	-	-	**2**
	2	-	-	-	2	2	2	2	n/a	-	**2**	2	2	2	2	1	2	**2**	**2**
	3	-	-	-	2	2	2	2	2	-	**2**	2	2	2	2	2	2	**2**	**2**
	4	2	2	2	2	2	2	2	2	-	**2**	2	2	2	2	2	2	**2**	**2**
	5	-	-	-	2	-	-	-	-	-	**-**	-	-	2	-	-	-	**-**	**2**
	6	-	-	-	2	-	-	-	-	-	**-**	-	-	2	-	-	-	**-**	**2**
	7	2	2	2	2	2	2	2	2	-	**2**	2	2	2	2	2	2	**2**	**2**
	8	-	-	-	-	-	-	-	-	-	**-**	-	1	-	-	-	-	**-**	**1**
																	Grand Total		**3**

STN130

Year		2001	2002			2003													Overall
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Total
Age class		A	A	J	J	A					A	J						J	
Site	1	10	-	-	-	-	9	10	8	n/a	**10**	-	-	-	-	-	-	-	**14**
	2	-	-	-	10	7	9	14	n/a	-	**14**	11	10	11	2	3	2	**11**	**17**
	3	-	-	-	11	7	8	11	5	-	**11**	7	13	13	9	8	7	**13**	**17**
	4	13	7	10	10	8	8	8	4	-	**8**	4	9	11	8	9	6	**11**	**17**
	5	-	-	-	5	-	-	-	-	-	**-**	-	-	4	-	-	-	**-**	**6**
	6	-	-	-	5	-	-	-	-	-	**-**	-	-	4	-	-	-	**-**	**7**
	7	8	1	1	4	4	2	5	4	-	**5**	1	5	3	5	2	6	**6**	**12**
	8	-	-	-	-	-	-	-	-	-	**-**	-	5	-	-	-	-	**-**	**5**
																	Grand Total		**20**

STN152

Year		2001	2002			2003													Overall
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Total
Age class		A	A	J	J	A					A	J						J	
Site	1	17	-	-	-	-	19	21	19	n/a	**21**	-	-	-	-	-	-	-	**38**
	2	-	-	-	34	14	32	33	n/a	-	**33**	29	31	19	7	5	5	**31**	**56**
	3	-	-	-	29	20	28	34	8	-	**34**	24	31	27	18	19	13	**31**	**59**
	4	40	16	22	29	25	29	25	11	-	**29**	21	28	28	20	20	24	**28**	**58**
	5	-	-	-	17	-	-	-	-	-	**-**	-	-	17	-	-	-	**-**	**23**
	6	-	-	-	19	-	-	-	-	-	**-**	-	-	19	-	-	-	**-**	**22**
	7	16	14	7	16	15	18	17	15	-	**18**	10	15	18	15	13	14	**18**	**35**
	8	-	-	-	-	-	-	-	-	-	**-**	-	13	-	-	-	-	**-**	**13**
																	Grand Total		**62**

STN208

Year	2001	2002			2003													Overall
Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Total
Age class	A	A	J	J	A					A	J						J	
Site 1	14	-	-	-	-	13	13	12	n/a	**13**	-	-	-	-	-	-	**-**	**21**
Site 2	-	-	-	19	13	22	26	n/a	-	**26**	17	25	22	8	4	6	**25**	**33**
Site 3	-	-	-	16	22	21	23	8	-	**23**	21	28	21	20	17	14	**28**	**32**
Site 4	27	16	20	22	21	23	21	11	-	**23**	14	20	18	12	20	16	**20**	**35**
Site 5	-	-	-	14	-	-	-	-	-	**-**	-	-	13	-	-	-	**-**	**17**
Site 6	-	-	-	13	-	-	-	-	-	**-**	-	-	12	-	-	-	**-**	**14**
Site 7	17	11	7	16	11	12	13	13	-	**13**	10	12	14	11	12	13	**14**	**20**
Site 8	-	-	-	-	-	-	-	-	-	**-**	-	12	-	-	-	-	**-**	**12**
																Grand Total		**37**

STN26

Year	2001	2002			2003													Overall
Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Total
Age class	A	A	J	J	A					A	J						J	
Site 1	7	-	-	-	-	7	7	7	n/a	**7**	-	-	-	-	-	-	**-**	**7**
Site 2	-	-	-	7	7	6	9	n/a	-	**9**	8	7	8	5	4	3	**8**	**11**
Site 3	-	-	-	6	6	7	8	4	-	**8**	5	7	7	7	5	3	**7**	**8**
Site 4	9	6	6	6	7	8	7	4	-	**8**	5	7	5	6	7	6	**7**	**11**
Site 5	-	-	-	3	-	-	-	-	-	**-**	-	-	3	-	-	-	**-**	**3**
Site 6	-	-	-	3	-	-	-	-	-	**-**	-	-	3	-	-	-	**-**	**3**
Site 7	4	3	3	3	3	2	2	3	-	**3**	1	3	3	3	2	3	**3**	**4**
Site 8	-	-	-	-	-	-	-	-	-	**-**	-	3	-	-	-	-	**-**	**3**
																Grand Total		**11**

STN9

Year	2001	2002			2003													Overall
Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Total
Age class	A	A	J	J	A					A	J						J	
Site 1	8	-	-	-	-	10	11	11	n/a	**11**	-	-	-	-	-	-	**-**	**13**
Site 2	-	-	-	12	8	11	12	n/a	-	**12**	14	15	10	6	5	4	**15**	**16**
Site 3	-	-	-	11	12	13	13	6	-	**13**	10	11	12	11	9	8	**12**	**15**
Site 4	14	9	12	14	10	14	9	5	-	**14**	10	9	9	9	10	9	**10**	**18**
Site 5	-	-	-	7	-	-	-	-	-	**-**	-	-	5	-	-	-	**-**	**8**
Site 6	-	-	-	8	-	-	-	-	-	**-**	-	-	8	-	-	-	**-**	**8**
Site 7	7	5	4	7	5	8	8	6	-	**8**	5	7	8	6	6	6	**8**	**11**
Site 8	-	-	-	-	-	-	-	-	-	**-**	-	10	-	-	-	-	**-**	**10**
																Grand Total		**18**

STN94

Year		2001	2002			2003													Overall
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Total
Age class		A	A	J	J	A					A	J						J	
Site	1	8	-	-	-	-	7	8	7	n/a	**8**	-	-	-	-	-	-	-	**12**
	2	-	-	-	8	9	10	10	n/a	-	**10**	10	12	7	5	2	3	**12**	**18**
	3	-	-	-	9	7	7	14	4	-	**14**	10	9	12	9	7	6	**12**	**19**
	4	14	8	9	9	10	12	11	4	-	**12**	6	8	11	5	8	8	**11**	**21**
	5	-	-	-	4	-	-	-	-	-	**-**	-	-	4	-	-	-	**-**	**5**
	6	-	-	-	4	-	-	-	-	-	**-**	-	-	3	-	-	-	**-**	**4**
	7	5	4	2	4	4	5	5	5	-	**5**	3	5	3	3	4	4	**5**	**6**
	8	-	-	-	-	-	-	-	-	-	**-**	-	3	-	-	-	-	**-**	**3**
																	Grand Total		**23**

STN96

Year		2001	2002			2003													Overall
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Total	Jul	Aug	Sep	Oct	Nov	Dec	Total	Total
Age class		A	A	J	J	A					A	J						J	
Site	1	11	-	-	-	-	10	8	13	n/a	**13**	-	-	-	-	-	-	-	**15**
	2	-	-	-	13	9	11	13	n/a	-	**13**	13	11	12	3	3	3	**13**	**17**
	3	-	-	-	12	11	12	16	6	-	**16**	11	11	12	11	10	8	**12**	**18**
	4	15	10	11	12	9	11	11	5	-	**11**	6	10	10	10	11	10	**11**	**21**
	5	-	-	-	8	-	-	-	-	-	**-**	-	-	5	-	-	-	**-**	**8**
	6	-	-	-	6	-	-	-	-	-	**-**	-	-	6	-	-	-	**-**	**6**
	7	7	5	4	6	7	6	6	5	-	**7**	5	7	6	5	5	6	**7**	**8**
	8	-	-	-	-	-	-	-	-	-	**-**	-	3	-	-	-	-	**-**	**3**
																	Grand Total		**22**

Table 3. Allelic richness of each sample from each site at each locus. Allelic Richness (FSTAT vers 2.9.3.1, Goudet 2002) is a measure of the number of alleles in a sample after adjusting for sample size and therefore allows comparisons across samples.

ATP1a2

	Year	2001	2002			2003												Pooled
	Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Max	Jul	Aug	Sep	Oct	Nov	Dec	Max	Richness
	Age class	A	A	J	J	A					A	J						J	
Site	1	1.98	-	-	-	-	1.25	1.15	1.00	n/a	**1.25**	-	-	-	-	-	-	-	**2.00**
	2	-	-	-	1.90	1.93	1.90	1.61	n/a	-	**1.93**	1.86	1.86	1.98	1.00	2.00	1.75	**2.00**	**2.00**
	3	-	-	-	1.65	1.86	1.73	1.83	2.00	-	**2.00**	1.91	1.96	1.95	1.97	1.98	1.97	**1.98**	**2.00**
	4	1.97	1.97	1.95	1.90	1.74	1.95	1.94	1.99	-	**1.99**	1.90	1.78	1.78	1.84	1.91	1.98	**1.98**	**2.00**
	5	-	-	-	1.25	-	-	-	-	-	-	-	-	1.40	-	-	-	-	**2.00**
	6	-	-	-	1.19	-	-	-	-	-	-	-	-	1.36	-	-	-	-	**2.00**
	7	1.31	1.27	1.00	1.35	1.59	1.08	1.22	1.08	-	**1.59**	1.00	1.27	1.30	1.42	1.28	1.28	**1.42**	**2.00**
	8	-	-	-	-	-	-	-	-	-	-	-	1.00	-	-	-	-	-	**1.00**

MyoHC

	Year	2001	2002			2003												Pooled
	Month	Jul	Jul	Jul	Sep	Richness	May	Jun	Jul	Aug	Max	Jul	Aug	Sep	Oct	Nov	Dec	Max	Richness
	Age class	A	A	J	J	A					A	J						J	
Site	1	1.73	-	-	-	-	1.67	1.65	1.94	n/a	**1.94**	-	-	-	-	-	-	-	**2.00**
	2	-	-	-	1.89	1.90	1.92	1.83	n/a	-	**1.92**	1.91	1.69	1.83	1.97	2.00	2.00	**2.00**	**2.00**
	3	-	-	-	1.96	1.88	1.88	1.87	1.60	-	**1.88**	1.93	1.90	1.92	1.93	1.88	1.77	**1.93**	**2.00**
	4	1.86	1.88	1.93	1.97	1.97	1.89	1.88	1.97	-	**1.97**	1.96	1.96	1.92	1.96	1.95	1.94	**1.96**	**2.00**
	5	-	-	-	1.96	-	-	-	-	-	-	-	-	1.96	-	-	-	-	**2.00**
	6	-	-	-	1.97	-	-	-	-	-	-	-	-	1.97	-	-	-	-	**2.00**
	7	1.97	1.98	2.00	1.97	1.97	1.98	1.94	1.97	-	**1.98**	1.96	1.96	1.97	1.93	1.97	1.94	**1.97**	**2.00**
	8	-	-	-	-	-	-	-	-	-	-	-	1.95	-	-	-	-	-	**2.00**

bAR2

	Year	2001	2002			2003												Pooled
	Month	Jul	Jul	Jul	Sep	Richness	May	Jun	Jul	Aug	Max	Jul	Aug	Sep	Oct	Nov	Dec	Max	Richness
	Age class	A	A	J	J						A	J						J	
Site	1	1.00	-	-	-	-	1.00	1.00	1.00	n/a	**1.00**	-	-	-	-	-	-	-	**1.00**
	2	-	-	-	1.84	1.53	1.35	1.17	n/a	-	**1.53**	1.39	1.23	1.46	1.87	2.00	1.75	**2.00**	**2.00**
	3	-	-	-	1.54	1.35	1.20	1.40	1.87	-	**1.87**	1.09	1.41	1.69	1.48	1.42	1.61	**1.69**	**2.00**
	4	1.31	1.31	1.39	1.49	1.78	1.52	1.48	1.85	-	**1.85**	1.54	1.77	1.82	1.86	1.76	1.75	**1.86**	**2.00**
	5	-	-	-	1.92	-	-	-	-	-	-	-	-	1.82	-	-	-	-	**2.00**
	6	-	-	-	1.85	-	-	-	-	-	-	-	-	1.87	-	-	-	-	**2.00**
	7	1.71	1.83	1.97	1.91	1.86	1.87	1.87	1.95	-	**1.95**	1.98	1.96	1.96	1.86	1.88	1.94	**1.98**	**2.00**
	8	-	-	-	-	-	-	-	-	-	-	-	1.81	-	-	-	-	-	**2.00**

Cytb

	Year	2001	2002			2003												Pooled
	Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Max	Jul	Aug	Sep	Oct	Nov	Dec	Max	Richness
	Age class	A	A	J	J	A					A	J						J	
Site	1	1.00	-	-	-	-	1.17	1.28	1.00	n/a	**1.28**	-	-	-	-	-	-	-	**1.96**
	2	-	-	-	1.95	1.64	1.73	1.40	n/a	-	**1.73**	1.49	1.23	1.64	1.87	1.00	1.96	**1.96**	**2.00**
	3	-	-	-	1.96	1.59	1.26	1.66	2.00	-	**2.00**	1.73	1.66	1.90	1.76	1.83	1.61	**1.90**	**2.00**
	4	1.76	1.70	1.79	1.97	1.97	1.85	1.69	1.69	-	**1.97**	1.97	1.96	1.97	1.92	1.92	1.98	**1.98**	**2.00**
	5	-	-	-	1.85	-	-	-	-	-	-	-	-	1.92	-	-	-	-	**2.00**
	6	-	-	-	1.82	-	-	-	-	-	-	-	-	1.75	-	-	-	-	**2.00**
	7	1.88	1.83	2.00	1.79	1.92	1.84	1.78	1.49	-	**1.92**	1.96	1.87	1.80	1.68	1.35	1.81	**1.96**	**2.00**
	8	-	-	-	-	-	-	-	-	-	-	-	1.00	-	-	-	-	-	**1.00**

STN130

	Year	2001	2002			2003												Pooled
	Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Max	Jul	Aug	Sep	Oct	Nov	Dec	Max	Richness
	Age class	A	A	J	J	A					A	J						J	
Site	1	3.73	-	-	-	-	2.89	3.00	3.40	n/a	**3.40**	-	-	-	-	-	-	-	**12.42**
	2	-	-	-	2.16	3.22	2.95	3.33	n/a	-	**3.33**	2.86	2.78	2.81	1.87	3.00	1.75	**3.00**	**11.62**
	3	-	-	-	2.44	2.35	2.72	2.83	3.40	-	**3.40**	2.13	2.77	2.35	1.98	2.80	2.37	**2.80**	**10.62**
	4	2.94	3.08	2.91	2.38	2.03	2.61	2.56	2.55	-	**2.61**	1.93	1.86	2.30	2.14	2.07	2.11	**2.30**	**10.01**
	5	-	-	-	1.51	-	-	-	-	-	-	-	-	1.18	-	-	-	-	**4.54**
	6	-	-	-	1.56	-	-	-	-	-	-	-	-	1.44	-	-	-	-	**4.83**
	7	1.70	1.00	1.00	1.38	1.39	1.23	1.35	1.37	-	**1.39**	1.00	1.30	1.26	1.58	1.19	1.68	**1.68**	**4.55**
	8	-	-	-	-	-	-	-	-	-	-	-	1.47	-	-	-	-	-	**5.00**

STN152

	Year	2001	2002			2003												Pooled
	Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Max	Jul	Aug	Sep	Oct	Nov	Dec	Max	Richness
	Age class	A	A	J	J	A					A	J						J	
Site	1	5.24	-	-	-	-	4.96	5.41	5.40	n/a	**5.41**	-	-	-	-	-	-	-	**31.03**
	2	-	-	-	5.16	5.20	5.44	5.30	n/a	-	**5.44**	5.31	5.36	5.12	4.83	5.00	4.21	**5.36**	**33.52**
	3	-	-	-	4.83	5.20	5.25	5.33	5.33	-	**5.33**	5.25	5.22	4.88	4.88	5.53	4.97	**5.53**	**32.00**
	4	5.22	5.18	4.92	5.29	4.54	5.32	5.25	5.51	-	**5.51**	5.13	5.03	4.96	4.58	4.87	5.13	**5.13**	**31.65**
	5	-	-	-	4.13	-	-	-	-	-	-	-	-	3.98	-	-	-	-	**17.79**
	6	-	-	-	4.39	-	-	-	-	-	-	-	-	4.33	-	-	-	-	**18.70**
	7	4.37	4.64	4.52	4.48	4.20	4.41	4.38	4.41	-	**4.41**	4.36	4.27	4.65	4.50	4.14	4.52	**4.65**	**18.67**
	8	-	-	-	-	-	-	-	-	-	-	-	3.54	-	-	-	-	-	**13.00**

STN208

	Year	2001	2002			2003													Pooled
	Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Max	Jul	Aug	Sep	Oct	Nov	Dec	Max	Richness
	Age class	A	A	J	J	A					A	J						J	
Site	1	4.56	-	-	-	-	4.55	4.86	4.66	n/a	**4.86**	-	-	-	-	-	-	-	**18.58**
	2	-	-	-	4.84	4.74	5.25	5.11	n/a	-	**5.25**	4.73	4.96	5.17	5.33	4.00	4.93	**5.33**	**23.82**
	3	-	-	-	4.77	5.37	5.02	4.89	5.33	-	**5.37**	5.13	5.30	4.98	5.06	5.47	4.53	**5.47**	**23.45**
	4	5.08	5.12	5.07	4.88	4.67	4.97	5.21	5.51	-	**5.51**	4.39	4.83	4.43	4.23	4.98	4.93	**4.98**	**21.83**
	5	-	-	-	3.98	-	-	-	-	-	-	-	-	4.25	-	-	-	-	**14.04**
	6	-	-	-	4.06	-	-	-	-	-	-	-	-	4.05	-	-	-	-	**12.76**
	7	4.15	4.10	4.52	4.52	3.88	4.02	4.00	4.45	-	**4.45**	4.63	4.12	4.17	4.03	4.51	4.60	**4.63**	**13.84**
	8	-	-	-	-	-	-	-	-	-	-	-	3.90	-	-	-	-	-	**12.00**

STN26

	Year	2001	2002			2003													Pooled
	Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Max	Jul	Aug	Sep	Oct	Nov	Dec	Max	Richness
	Age class	A	A	J	J	A					A	J						J	
Site	1	3.47	-	-	-	-	3.60	4.04	4.19	n/a	**4.19**	-	-	-	-	-	-	-	**7.00**
	2	-	-	-	3.14	3.99	3.63	3.78	n/a	-	**3.99**	3.56	3.68	3.72	3.93	4.00	2.71	**4.00**	**8.02**
	3	-	-	-	2.53	3.53	3.55	3.53	3.07	-	**3.55**	3.33	3.62	3.04	3.40	3.10	2.70	**3.62**	**7.01**
	4	3.43	3.35	3.26	2.79	2.50	2.89	3.51	2.97	-	**3.51**	2.48	2.95	2.08	2.49	2.88	3.23	**3.23**	**7.79**
	5	-	-	-	2.09	-	-	-	-	-	-	-	-	1.45	-	-	-	-	**3.00**
	6	-	-	-	1.52	-	-	-	-	-	-	-	-	1.60	-	-	-	-	**2.95**
	7	1.75	1.83	2.47	1.41	1.45	1.23	1.22	1.83	-	**1.83**	1.00	1.39	1.53	1.70	1.48	1.64	**1.70**	**3.06**
	8	-	-	-	-	-	-	-	-	-	-	-	1.24	-	-	-	-	-	**3.00**

STN9

	Year	2001	2002			2003													Pooled
	Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Max	Jul	Aug	Sep	Oct	Nov	Dec	Max	Richness
	Age class	A	A	J	J	A					A	J						J	
Site	1	3.59	-	-	-	-	4.16	4.68	4.62	n/a	**4.68**	-	-	-	-	-	-	-	**12.02**
	2	-	-	-	4.37	3.65	3.94	4.45	n/a	-	**4.45**	4.13	4.40	4.05	4.26	5.00	3.68	**5.00**	**12.87**
	3	-	-	-	4.13	4.32	4.54	4.47	4.26	-	**4.54**	4.21	4.08	4.04	3.95	4.61	4.32	**4.61**	**12.26**
	4	4.42	4.54	4.37	4.23	3.73	4.50	4.17	3.65	-	**4.50**	4.24	3.84	3.59	3.87	4.04	3.62	**4.24**	**11.89**
	5	-	-	-	3.24	-	-	-	-	-	-	-	-	3.21	-	-	-	-	**7.56**
	6	-	-	-	3.45	-	-	-	-	-	-	-	-	3.52	-	-	-	-	**7.62**
	7	3.44	3.06	3.18	3.45	3.12	3.28	3.37	3.34	-	**3.37**	2.95	3.25	3.50	3.10	3.49	3.18	**3.50**	**7.03**
	8	-	-	-	-	-	-	-	-	-	-	-	3.39	-	-	-	-	-	**10.00**

STN94

	Year	2001	2002			2003												Pooled
	Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Max	Jul	Aug	Sep	Oct	Nov	Dec	Max	Richness
	Age class	A	A	J	J	A					A	J						J	
Site	1	3.83	-	-	-	-	3.72	3.67	3.85	n/a	**3.85**	-	-	-	-	-	-	-	**10.56**
	2	-	-	-	3.20	4.09	3.48	3.62	n/a	-	**4.09**	3.59	3.69	3.17	4.00	2.00	2.71	**4.00**	**10.77**
	3	-	-	-	3.19	3.70	3.39	3.76	3.43	-	**3.76**	3.67	3.10	3.36	3.33	3.95	2.98	**3.95**	**10.33**
	4	3.73	3.83	3.76	3.29	3.31	3.58	3.55	3.05	-	**3.58**	2.61	3.19	2.76	2.50	3.03	3.16	**3.19**	**11.01**
	5	-	-	-	2.16	-	-	-	-	-	-	-	-	2.35	-	-	-	-	**4.66**
	6	-	-	-	2.14	-	-	-	-	-	-	-	-	2.14	-	-	-	-	**3.50**
	7	2.46	2.68	1.99	2.42	2.33	2.29	2.20	2.57	-	**2.57**	2.25	2.52	2.27	2.15	2.42	2.26	**2.52**	**4.48**
	8	-	-	-	-	-	-	-	-	-	-	-	2.27	-	-	-	-	-	**3.00**

STN96

	Year	2001	2002			2003												Pooled
	Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Max	Jul	Aug	Sep	Oct	Nov	Dec	Max	Richness
	Age class	A	A	J	J	A					A	J						J	
Site	1	4.61	-	-	-	-	4.26	4.00	5.03	n/a	**5.03**	-	-	-	-	-	-	-	**13.05**
	2	-	-	-	4.17	4.25	4.14	4.26	n/a	-	**4.26**	3.97	4.19	4.24	2.86	3.00	2.71	**4.24**	**12.98**
	3	-	-	-	3.54	4.30	4.08	4.19	4.50	-	**4.50**	4.05	4.13	3.86	3.96	4.59	4.19	**4.59**	**12.69**
	4	3.90	4.26	4.12	3.92	3.63	3.96	4.08	3.79	-	**4.08**	3.30	3.30	3.36	4.08	4.00	4.27	**4.27**	**11.56**
	5	-	-	-	3.02	-	-	-	-	-	-	-	-	2.72	-	-	-	-	**7.22**
	6	-	-	-	3.01	-	-	-	-	-	-	-	-	3.04	-	-	-	-	**6.00**
	7	3.34	2.84	3.49	2.69	3.20	2.82	3.37	2.77	-	**3.37**	3.03	3.01	3.17	2.64	2.95	3.12	**3.17**	**6.26**
	8	-	-	-	-	-	-	-	-	-	-	-	2.33	-	-	-	-	-	**3.00**

Table 4. F_{IS} values at each locus in each sample each month.

ATP1a2

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.52	-	-	-	-	-0.03	0.00	n/a	n/a	**-0.02**	-	-	-	-	-	-	-
	2	-	-	-	-0.13	0.85	0.69	0.51	n/a	-	**0.68**	0.17	-0.06	0.13	n/a	-0.33	0.00	**-0.02**
	3	-	-	-	-0.17	0.56	0.70	0.53	1.00	-	**0.70**	0.00	0.01	-0.31	-0.06	-0.39	-0.36	**-0.18**
	4	0.52	0.00	0.42	-0.15	-0.23	-0.50	-0.57	-0.09	-	**-0.35**	-0.40	-0.04	-0.16	-0.14	-0.29	-0.29	**-0.22**
	5	-	-	-	-0.04	-	-	-	-	-	**-**	-	-	0.20	-	-	-	-
	6	-	-	-	-0.02	-	-	-	-	-	**-**	-	-	-0.07	-	-	-	-
	7	0.30	-0.03	n/a	-0.06	0.15	0.00	-0.03	0.00	-	**0.03**	n/a	0.38	-0.05	0.36	-0.04	-0.04	**0.12**
	8	-	-	-	-	-	-	-	-	-	**-**	-	n/a	-	-	-	-	-

MyoHC

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.15	-	-	-	-	-0.18	0.24	0.06	n/a	**0.04**	-	-	-	-	-	-	-
	2	-	-	-	0.11	-0.01	0.01	-0.11	n/a	-	**-0.04**	0.13	0.09	0.51	0.60	-0.33	-1.00	**0.00**
	3	-	-	-	0.05	0.27	0.08	0.02	0.00	-	**0.09**	0.05	0.12	0.14	0.07	0.06	0.20	**0.11**
	4	0.12	0.47	0.41	-0.08	-0.08	0.22	0.17	0.14	-	**0.11**	0.35	-0.26	-0.06	-0.29	-0.18	0.44	**0.00**
	5	-	-	-	-0.08	-	-	-	-	-	**-**	-	-	0.09	-	-	-	-
	6	-	-	-	0.30	-	-	-	-	-	**-**	-	-	0.16	-	-	-	-
	7	0.54	0.07	0.41	-0.21	0.34	0.29	0.02	-0.02	-	**0.16**	-0.13	-0.19	0.05	-0.08	0.01	-0.13	**-0.08**
	8	-	-	-	-	-	-	-	-	-	**-**	-	0.03	-	-	-	-	-

bAR2

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	n/a	-	-	-	-	n/a	n/a	n/a	n/a	n/a	-	-	-	-	-	-	-
	2	-	-	-	0.31	-0.08	-0.06	-0.02	n/a	-	**-0.05**	-0.07	-0.03	0.36	1.00	-0.33	0.00	**0.15**
	3	-	-	-	-0.12	-0.05	0.66	0.20	-0.14	-	**0.17**	0.00	-0.08	0.23	0.28	1.00	0.45	**0.31**
	4	0.74	-0.03	-0.07	0.12	-0.04	0.11	0.22	0.63	-	**0.23**	0.78	-0.02	0.02	0.16	-0.23	-0.22	**0.08**
	5	-	-	-	0.10	-	-	-	-	-	**-**	-	-	0.02	-	-	-	-
	6	-	-	-	-0.24	-	-	-	-	-	**-**	-	-	0.22	-	-	-	-
	7	-0.07	-0.03	0.33	-0.06	0.33	-0.23	-0.08	-0.07	-	**-0.01**	0.47	0.11	0.03	0.33	0.44	-0.13	**0.21**
	8	-	-	-	-	-	-	-	-	-	**-**	-	-0.07	-	-	-	-	-

STN130

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.09	-	-	-	-	-0.03	0.05	0.01	n/a	**0.01**	-	-	-	-	-	-	-
	2	-	-	-	0.16	0.27	-0.02	0.04	n/a	-	**0.10**	-0.01	0.01	0.02	-0.14	0.50	0.00	**0.06**
	3	-	-	-	-0.01	0.17	-0.02	0.13	0.11	-	**0.10**	-0.13	0.00	0.01	0.02	-0.02	-0.13	**-0.04**
	4	-0.04	-0.18	-0.09	-0.01	-0.05	0.20	-0.07	-0.17	-	**-0.02**	-0.13	0.06	0.06	-0.12	-0.10	-0.16	**-0.07**
	5	-	-	-	0.21	-	-	-	-	-	**-**	-	-	-0.01	-	-	-	**-**
	6	-	-	-	0.18	-	-	-	-	-	**-**	-	-	0.08	-	-	-	**-**
	7	0.11	n/a	n/a	-0.04	-0.03	-0.03	-0.03	-0.04	-	**-0.03**	n/a	-0.02	-0.03	0.12	-0.02	0.09	**0.03**
	8	-	-	-	-	-	-	-	-	-	**-**	-	-0.05	-	-	-	-	**-**

STN152

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.39	-	-	-	-	0.30	0.33	0.36	n/a	**0.33**	-	-	-	-	-	-	-
	2	-	-	-	0.17	0.25	0.36	0.37	n/a	-	**0.32**	0.27	0.30	0.41	0.37	0.33	0.46	**0.36**
	3	-	-	-	0.20	0.41	0.40	0.39	0.18	-	**0.34**	0.47	0.24	0.40	0.21	0.13	0.36	**0.30**
	4	0.29	0.32	0.25	0.25	0.32	0.35	0.19	0.43	-	**0.32**	0.13	0.14	0.11	0.13	0.31	0.16	**0.16**
	5	-	-	-	0.09	-	-	-	-	-	**-**	-	-	-0.04	-	-	-	**-**
	6	-	-	-	0.09	-	-	-	-	-	**-**	-	-	0.13	-	-	-	**-**
	7	0.11	0.04	0.26	-0.04	-0.07	-0.03	0.10	-0.01	-	**0.00**	-0.06	-0.04	0.11	-0.04	0.12	-0.07	**0.00**
	8	-	-	-	-	-	-	-	-	-	**-**	-	0.19	-	-	-	-	**-**

STN208

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	-0.09	-	-	-	-	-0.03	0.18	0.02	n/a	**0.06**	-	-	-	-	-	-	-
	2	-	-	-	0.05	-0.04	0.15	0.01	n/a	-	**0.04**	0.14	-0.02	-0.03	-0.05	-0.20	-0.09	**-0.04**
	3	-	-	-	0.02	0.09	-0.03	0.06	0.18	-	**0.08**	0.07	-0.01	0.09	-0.04	0.04	0.00	**0.02**
	4	0.10	-0.08	-0.04	0.04	0.08	0.04	0.00	-0.04	-	**0.02**	0.06	0.03	0.08	0.30	0.06	0.01	**0.09**
	5	-	-	-	0.14	-	-	-	-	-	**-**	-	-	0.06	-	-	-	**-**
	6	-	-	-	0.06	-	-	-	-	-	**-**	-	-	0.08	-	-	-	**-**
	7	0.17	0.10	-0.15	0.12	0.04	0.19	0.09	0.03	-	**0.09**	0.08	0.14	0.00	-0.02	-0.03	0.10	**0.05**
	8	-	-	-	-	-	-	-	-	-	**-**	-	-0.01	-	-	-	-	**-**

STN26

Year	2001	2002			2003												
Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class	A	A	J	J	A					A	J						J
Site 1	0.01	-	-	-	-	0.15	-0.02	0.20	n/a	**0.11**	-	-	-	-	-	-	-
Site 2	-	-	-	0.26	0.49	0.48	0.18	n/a	-	**0.38**	0.24	0.20	0.19	0.29	0.67	0.20	**0.30**
Site 3	-	-	-	0.34	0.27	0.34	0.45	0.41	-	**0.37**	0.41	0.27	0.31	0.28	0.24	0.49	**0.34**
Site 4	0.40	0.23	0.22	0.44	0.33	0.45	0.31	0.36	-	**0.36**	0.47	0.62	0.45	0.26	0.06	0.15	**0.33**
Site 5	-	-	-	0.17	-	-	-	-	-	-	-	-	-0.08	-	-	-	-
Site 6	-	-	-	-0.01	-	-	-	-	-	-	-	-	0.15	-	-	-	-
Site 7	0.16	0.12	0.08	0.24	0.17	-0.03	-0.03	0.24	-	**0.09**	-0.01	0.15	-0.01	0.06	0.14	0.26	**0.10**
Site 8	-	-	-	-	-	-	-	-	-	-	-	-0.02	-	-	-	-	-

STN9

Year	2001	2002			2003												
Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class	A	A	J	J	A					A	J						J
Site 1	-0.09	-	-	-	-	-0.09	0.05	0.02	n/a	**-0.01**	-	-	-	-	-	-	-
Site 2	-	-	-	-0.03	-0.07	0.01	0.02	n/a	-	**-0.02**	-0.02	0.03	0.02	-0.21	-0.09	0.10	**-0.03**
Site 3	-	-	-	0.13	0.05	0.05	0.02	0.06	-	**0.04**	-0.04	0.11	0.02	0.00	-0.04	-0.16	**-0.02**
Site 4	-0.06	-0.07	0.08	-0.01	0.04	0.11	0.19	0.09	-	**0.11**	-0.07	0.01	0.02	-0.04	-0.01	0.16	**0.01**
Site 5	-	-	-	0.01	-	-	-	-	-	-	-	-	-0.02	-	-	-	-
Site 6	-	-	-	-0.15	-	-	-	-	-	-	-	-	0.06	-	-	-	-
Site 7	-0.02	0.06	0.56	0.00	0.08	0.11	-0.09	-0.09	-	**0.00**	-0.39	-0.04	0.04	0.22	-0.18	0.08	**-0.04**
Site 8	-	-	-	-	-	-	-	-	-	-	-	-0.01	-	-	-	-	-

STN94

Year	2001	2002			2003												
Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class	A	A	J	J	A					A	J						J
Site 1	0.28	-	-	-	-	0.11	0.24	-0.08	n/a	**0.09**	-	-	-	-	-	-	-
Site 2	-	-	-	0.11	0.33	0.18	0.28	n/a	-	**0.26**	0.01	0.18	0.17	0.56	-0.33	0.20	**0.13**
Site 3	-	-	-	0.18	-0.05	0.14	0.22	0.52	-	**0.20**	0.07	0.10	0.16	0.15	0.17	-0.05	**0.10**
Site 4	0.28	0.15	0.39	0.23	0.22	0.25	0.44	0.40	-	**0.33**	0.56	0.21	0.18	0.11	0.02	0.24	**0.22**
Site 5	-	-	-	-0.17	-	-	-	-	-	-	-	-	-0.19	-	-	-	-
Site 6	-	-	-	0.30	-	-	-	-	-	-	-	-	0.18	-	-	-	-
Site 7	0.29	-0.38	0.06	0.04	0.18	0.01	0.16	0.02	-	**0.09**	0.37	0.29	-0.02	-0.01	-0.03	0.04	**0.11**
Site 8	-	-	-	-	-	-	-	-	-	-	-	-0.12	-	-	-	-	-

STN96																	
Year		2001	2002			2003											
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.18	-	-	-	-	0.00	-0.15	-0.08	n/a	**-0.08**	-	-	-	-	-	-	-
	2	-	-	-	0.10	-0.10	-0.06	0.09	n/a	-	**-0.02**	0.09	0.09	0.17	-0.14	-0.50	-0.29	**-0.10**
	3	-	-	-	-0.10	0.30	-0.03	0.12	-0.14	-	**0.06**	0.02	-0.03	-0.02	-0.02	-0.04	-0.10	**-0.03**
	4	0.03	-0.11	0.09	0.08	0.19	0.02	0.00	-0.25	-	**-0.01**	-0.13	-0.02	-0.07	-0.09	-0.02	0.19	**-0.02**
	5	-	-	-	0.12	-	-	-	-	-	-	-	-	0.08	-	-	-	-
	6	-	-	-	0.04	-	-	-	-	-	-	-	-	0.10	-	-	-	-
	7	0.06	-0.02	0.17	-0.04	0.05	0.23	-0.03	-0.09	-	**0.04**	-0.16	0.08	0.10	0.23	0.27	-0.01	**0.09**
	8	-	-	-	-	-	-	-	-	-	-	-	-0.08	-	-	-	-	-

Table 5. Heterozygosity (gene diversity, FSTAT Goudet 2002) per locus and sample.

ATP1a2

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.52	-	-	-	-	0.09	0.05	0.00	n/a	**0.05**	-	-	-	-	-	-	-
	2	-	-	-	0.43	0.47	0.44	0.25	n/a	-	**0.38**	0.40	0.40	0.51	0.00	0.50	0.25	**0.34**
	3	-	-	-	0.27	0.40	0.32	0.38	0.60	-	**0.42**	0.44	0.49	0.48	0.51	0.50	0.49	**0.48**
	4	0.51	0.50	0.49	0.43	0.32	0.47	0.47	0.52	-	**0.45**	0.42	0.35	0.35	0.38	0.44	0.50	**0.41**
	5	-	-	-	0.09	-	-	-	-	-	-	-	-	0.15	-	-	-	-
	6	-	-	-	0.07	-	-	-	-	-	-	-	-	0.13	-	-	-	-
	7	0.11	0.09	0.00	0.13	0.24	0.03	0.08	0.03	-	**0.09**	0.00	0.10	0.11	0.16	0.10	0.10	**0.09**
	8	-	-	-	-	-	-	-	-	-	-	-	0.00	-	-	-	-	-

MyoHC

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.31	-	-	-	-	0.28	0.26	0.47	n/a	**0.34**	-	-	-	-	-	-	-
	2	-	-	-	0.43	0.42	0.45	0.38	n/a	-	**0.42**	0.44	0.29	0.38	0.50	0.50	0.50	**0.43**
	3	-	-	-	0.49	0.42	0.41	0.41	0.20	-	**0.36**	0.46	0.43	0.45	0.46	0.41	0.33	**0.43**
	4	0.40	0.42	0.46	0.50	0.50	0.43	0.42	0.50	-	**0.46**	0.49	0.49	0.45	0.49	0.48	0.47	**0.48**
	5	-	-	-	0.49	-	-	-	-	-	-	-	-	0.49	-	-	-	-
	6	-	-	-	0.51	-	-	-	-	-	-	-	-	0.50	-	-	-	-
	7	0.51	0.51	0.57	0.50	0.50	0.51	0.47	0.50	-	**0.50**	0.48	0.49	0.50	0.46	0.50	0.47	**0.49**
	8	-	-	-	-	-	-	-	-	-	-	-	0.48	-	-	-	-	-

bAR2

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.00	-	-	-	-	0.00	0.00	0.00	n/a	**0.00**	-	-	-	-	-	-	-
	2	-	-	-	0.39	0.20	0.13	0.06	n/a	-	**0.13**	0.14	0.08	0.17	0.40	0.50	0.25	**0.26**
	3	-	-	-	0.21	0.13	0.07	0.15	0.35	-	**0.17**	0.03	0.15	0.29	0.18	0.15	0.24	**0.18**
	4	0.11	0.11	0.14	0.19	0.35	0.20	0.18	0.38	-	**0.28**	0.21	0.34	0.37	0.40	0.32	0.32	**0.33**
	5	-	-	-	0.46	-	-	-	-	-	-	-	-	0.37	-	-	-	-
	6	-	-	-	0.39	-	-	-	-	-	-	-	-	0.41	-	-	-	-
	7	0.30	0.37	0.50	0.44	0.40	0.41	0.41	0.48	-	**0.42**	0.52	0.49	0.49	0.40	0.42	0.47	**0.47**
	8	-	-	-	-	-	-	-	-	-	-	-	0.36	-	-	-	-	-

STN130

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.75	-	-	-	-	0.57	0.58	0.70	n/a	**0.61**	-	-	-	-	-	-	-
	2	-	-	-	0.37	0.68	0.58	0.67	n/a	-	**0.64**	0.56	0.54	0.53	0.35	0.67	0.25	**0.48**
	3	-	-	-	0.44	0.42	0.54	0.55	0.68	-	**0.55**	0.36	0.54	0.41	0.31	0.53	0.41	**0.43**
	4	0.58	0.61	0.57	0.43	0.33	0.50	0.48	0.49	-	**0.45**	0.32	0.28	0.40	0.36	0.33	0.38	**0.34**
	5	-	-	-	0.17	-	-	-	-	-	-	-	-	0.06	-	-	-	-
	6	-	-	-	0.19	-	-	-	-	-	-	-	-	0.15	-	-	-	-
	7	0.23	0.00	0.00	0.13	0.13	0.08	0.12	0.12	-	**0.11**	0.00	0.10	0.09	0.19	0.07	0.22	**0.11**
	8	-	-	-	-	-	-	-	-	-	-	-	0.16	-	-	-	-	-

STN152

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.96	-	-	-	-	0.92	0.97	0.97	n/a	**0.95**	-	-	-	-	-	-	-
	2	-	-	-	0.94	0.95	0.96	0.95	n/a	-	**0.96**	0.95	0.96	0.94	0.95	1.00	0.92	**0.95**
	3	-	-	-	0.91	0.95	0.95	0.95	0.98	-	**0.96**	0.95	0.94	0.92	0.92	0.97	0.94	**0.94**
	4	0.94	0.95	0.92	0.95	0.88	0.95	0.95	1.00	-	**0.94**	0.94	0.93	0.92	0.88	0.91	0.93	**0.92**
	5	-	-	-	0.83	-	-	-	-	-	-	-	-	0.81	-	-	-	-
	6	-	-	-	0.86	-	-	-	-	-	-	-	-	0.85	-	-	-	-
	7	0.86	0.89	0.90	0.87	0.84	0.86	0.86	0.87	-	**0.86**	0.86	0.85	0.89	0.87	0.83	0.87	**0.86**
	8	-	-	-	-	-	-	-	-	-	-	-	0.76	-	-	-	-	-

STN208

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.87	-	-	-	-	0.89	0.92	0.89	n/a	**0.90**	-	-	-	-	-	-	-
	2	-	-	-	0.91	0.89	0.95	0.93	n/a	-	**0.92**	0.90	0.92	0.94	0.95	0.83	0.92	**0.91**
	3	-	-	-	0.90	0.96	0.93	0.91	0.98	-	**0.94**	0.94	0.95	0.92	0.93	0.97	0.87	**0.93**
	4	0.93	0.93	0.93	0.91	0.89	0.92	0.94	0.96	-	**0.93**	0.86	0.91	0.87	0.85	0.92	0.92	**0.89**
	5	-	-	-	0.81	-	-	-	-	-	-	-	-	0.85	-	-	-	-
	6	-	-	-	0.82	-	-	-	-	-	-	-	-	0.83	-	-	-	-
	7	0.84	0.83	0.87	0.88	0.80	0.83	0.82	0.87	-	**0.83**	0.89	0.84	0.84	0.82	0.88	0.89	**0.86**
	8	-	-	-	-	-	-	-	-	-	-	-	0.81	-	-	-	-	-

STN26

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.77	-	-	-	-	0.78	0.83	0.86	n/a	**0.82**	-	-	-	-	-	-	-
	2	-	-	-	0.66	0.84	0.78	0.80	n/a	-	**0.81**	0.78	0.79	0.78	0.85	1.00	0.63	**0.80**
	3	-	-	-	0.54	0.78	0.78	0.76	0.68	-	**0.75**	0.74	0.77	0.66	0.74	0.71	0.66	**0.71**
	4	0.75	0.73	0.73	0.63	0.51	0.65	0.75	0.67	-	**0.64**	0.51	0.63	0.37	0.49	0.60	0.71	**0.55**
	5	-	-	-	0.41	-	-	-	-	-	**-**	-	-	0.15	-	-	-	**-**
	6	-	-	-	0.19	-	-	-	-	-	**-**	-	-	0.22	-	-	-	**-**
	7	0.26	0.30	0.63	0.15	0.16	0.08	0.08	0.30	-	**0.15**	0.00	0.13	0.19	0.25	0.19	0.24	**0.17**
	8	-	-	-	-	-	-	-	-	-	**-**	-	0.08	-	-	-	-	**-**

STN9

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.77	-	-	-	-	0.84	0.90	0.89	n/a	**0.87**	-	-	-	-	-	-	-
	2	-	-	-	0.87	0.73	0.82	0.87	n/a	-	**0.81**	0.83	0.86	0.83	0.83	0.92	0.83	**0.85**
	3	-	-	-	0.84	0.86	0.88	0.88	0.85	-	**0.87**	0.84	0.83	0.81	0.80	0.89	0.86	**0.84**
	4	0.87	0.89	0.87	0.84	0.77	0.88	0.85	0.79	-	**0.82**	0.85	0.80	0.77	0.81	0.83	0.77	**0.80**
	5	-	-	-	0.69	-	-	-	-	-	**-**	-	-	0.73	-	-	-	**-**
	6	-	-	-	0.75	-	-	-	-	-	**-**	-	-	0.76	-	-	-	**-**
	7	0.74	0.66	0.75	0.76	0.69	0.72	0.73	0.73	-	**0.72**	0.66	0.71	0.76	0.68	0.76	0.69	**0.71**
	8	-	-	-	-	-	-	-	-	-	**-**	-	0.73	-	-	-	-	**-**

STN94

Year		2001	2002			2003												
Month		Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class		A	A	J	J	A					A	J						J
Site	1	0.80	-	-	-	-	0.80	0.78	0.81	n/a	**0.80**	-	-	-	-	-	-	-
	2	-	-	-	0.71	0.85	0.76	0.78	n/a	-	**0.79**	0.78	0.78	0.71	0.90	0.50	0.63	**0.71**
	3	-	-	-	0.70	0.78	0.74	0.80	0.83	-	**0.79**	0.78	0.69	0.73	0.70	0.83	0.64	**0.73**
	4	0.78	0.81	0.80	0.72	0.72	0.77	0.76	0.71	-	**0.74**	0.62	0.70	0.64	0.60	0.68	0.69	**0.65**
	5	-	-	-	0.53	-	-	-	-	-	**-**	-	-	0.57	-	-	-	**-**
	6	-	-	-	0.52	-	-	-	-	-	**-**	-	-	0.53	-	-	-	**-**
	7	0.59	0.62	0.53	0.58	0.57	0.56	0.55	0.60	-	**0.57**	0.58	0.59	0.56	0.53	0.58	0.55	**0.56**
	8	-	-	-	-	-	-	-	-	-	**-**	-	0.55	-	-	-	-	**-**

STN96																	
Year	2001	2002			2003												
Month	Jul	Jul	Jul	Sep	Apr	May	Jun	Jul	Aug	Avg	Jul	Aug	Sep	Oct	Nov	Dec	Avg
Age class	A	A	J	J	A					A	J						J
Site 1	0.90	-	-	-	-	0.86	0.83	0.93	n/a	**0.87**	-	-	-	-	-	-	-
Site 2	-	-	-	0.84	0.85	0.84	0.85	n/a	-	**0.85**	0.82	0.85	0.85	0.70	0.67	0.58	**0.74**
Site 3	-	-	-	0.73	0.87	0.83	0.84	0.88	-	**0.85**	0.83	0.84	0.80	0.82	0.89	0.85	**0.84**
Site 4	0.81	0.84	0.84	0.81	0.77	0.82	0.83	0.80	-	**0.80**	0.73	0.72	0.73	0.83	0.82	0.86	**0.78**
Site 5	-	-	-	0.66	-	-	-	-	-	-	-	-	0.59	-	-	-	-
Site 6	-	-	-	0.65	-	-	-	-	-	-	-	-	0.67	-	-	-	-
Site 7	0.73	0.61	0.80	0.58	0.70	0.65	0.74	0.61	-	**0.67**	0.63	0.68	0.71	0.60	0.68	0.70	**0.67**
Site 8	-	-	-	-	-	-	-	-	-	-	-	0.51	-	-	-	-	-

Table 6. Ln likelihood values of there being K=[1-8] genetic clusters within the entire data set of 1961 individuals (see also Figure 5, Chapter 3) and the proportion of individuals from each of sites 1-8 in each of the 1-8 clusters. The most likely number of clusters in this data set is K=2 (see Chapter 3). A given cluster for one analysis of K does not necessarily correspond to the same cluster in an alternative analysis of K. See Table 5 for allele frequency divergence between each of the clusters.

K	Site	Ln(PrD|K)	Proportion of individuals in each of K=[1-8] clusters							
			1	2	3	4	5	6	7	8
1		-59649.7								
2	1	-54504.8	0.03	0.97						
	2		0.24	0.76						
	3		0.30	0.70						
	4		0.44	0.56						
	5		0.95	0.05						
	6		0.96	0.04						
	7		0.96	0.04						
	8		0.95	0.05						
3	1	-54491.8	0.93	0.04	0.04					
	2		0.71	0.14	0.15					
	3		0.64	0.19	0.17					
	4		0.50	0.25	0.25					
	5		0.04	0.49	0.48					
	6		0.03	0.48	0.49					
	7		0.03	0.48	0.49					
	8		0.04	0.39	0.57					
4	1	-54334.1	0.35	0.02	0.02	0.62				
	2		0.38	0.12	0.12	0.39				
	3		0.38	0.14	0.16	0.33				
	4		0.29	0.23	0.22	0.27				
	5		0.03	0.45	0.50	0.02				
	6		0.02	0.48	0.49	0.02				
	7		0.02	0.48	0.48	0.02				

	8		0.03	0.58	0.37	0.02				
5	**1**	-54087.3	0.02	0.02	0.43	0.51	0.02			
	2		0.10	0.10	0.39	0.34	0.07			
	3		0.12	0.10	0.34	0.33	0.11			
	4		0.16	0.16	0.26	0.27	0.16			
	5		0.34	0.31	0.02	0.02	0.30			
	6		0.35	0.33	0.02	0.02	0.29			
	7		0.32	0.36	0.02	0.02	0.28			
	8		0.18	0.17	0.02	0.02	0.61			
6	**1**	-54062.5	0.01	0.01	0.34	0.24	0.01	0.38		
	2		0.09	0.08	0.25	0.25	0.07	0.26		
	3		0.09	0.11	0.22	0.25	0.10	0.23		
	4		0.15	0.15	0.18	0.19	0.15	0.18		
	5		0.33	0.34	0.02	0.02	0.28	0.02		
	6		0.35	0.33	0.01	0.02	0.28	0.01		
	7		0.35	0.33	0.02	0.02	0.27	0.01		
	8		0.17	0.19	0.02	0.02	0.60	0.01		
7	**1**	-54053.1	0.01	0.37	0.01	0.23	0.01	0.35	0.01	
	2		0.07	0.23	0.05	0.26	0.08	0.25	0.07	
	3		0.09	0.20	0.08	0.24	0.07	0.23	0.09	
	4		0.12	0.17	0.12	0.18	0.12	0.18	0.12	
	5		0.28	0.02	0.22	0.02	0.24	0.01	0.22	
	6		0.26	0.01	0.21	0.02	0.25	0.01	0.23	
	7		0.23	0.01	0.20	0.01	0.27	0.01	0.25	
	8		0.14	0.02	0.54	0.02	0.13	0.01	0.14	
8	**1**	-53890.7	0.01	0.27	0.01	0.34	0.01	0.15	0.01	0.19
	2		0.07	0.19	0.05	0.17	0.06	0.18	0.07	0.21
	3		0.07	0.18	0.08	0.15	0.08	0.19	0.08	0.17
	4		0.12	0.14	0.11	0.12	0.11	0.15	0.11	0.13
	5		0.24	0.01	0.21	0.01	0.23	0.02	0.27	0.02
	6		0.24	0.01	0.21	0.01	0.23	0.01	0.27	0.02
	7		0.27	0.01	0.20	0.01	0.25	0.01	0.23	0.01
	8		0.13	0.01	0.53	0.02	0.15	0.01	0.14	0.01

Table 7. Allele frequency divergence between each of the clusters from structure simulations with vales of K=[1-8] for the entire data set of 1961 individuals (Chapter3). ** Allele frequency divergence is based on Kullback-Liebler D and provides a measure of genetic distance between populations (Pritchard, Stephens and Donnelly 2001). A large value of D indicates greater genetic distance than a small one. The most likely value of K for this data set was 2 (Chapter 3).

K		Allele frequency divergence (D)** between each of the clusters							
		1	2	3	4	5	6	7	8
1	1	-							
2	1	-							
	2	1.4	-						
3	1	-							
	2	0.8	-						
	3	0.9	0.1	-					
4	1	-							
	2	0.7	-						
	3	0.6	0.1	-					
	4	0.1	1.4	1.3	-				
5	1	-							
	2	0.1	-						
	3	1.3	1.5	-					
	4	1.3	1.4	0.1	-				
	5	0.1	0.1	0.8	0.8	-			
6	1	-							
	2	0.1	-						
	3	1.4	1.2	-					
	4	1.4	1.2	0.1	-				
	5	0.1	0.1	0.7	0.7	-			
	6	1.6	1.4	0.2	0.1	1.4	-		

7	1	-							
	2	1.2	-						
	3	0.2	0.8	-					
	4	1.3	0.1	1.4	-				
	5	0.2	0.9	0.2	0.8	-			
	6	1.4	0.2	1.5	0.1	1.6	-		
	7	0.1	0.7	0.2	0.7	0.1	0.8	-	
8	1	-							
	2	1.6	-						
	3	0.2	0.9	-					
	4	1.4	0.2	1.3	-				
	5	0.2	0.8	0.2	0.8	-			
	6	1.4	0.2	1.3	0.2	1.2	-		
	7	0.2	0.8	0.2	0.8	0.1	0.7	-	
	8	1.5	0.2	1.4	0.2	1.3	0.1	1.4	-

Table 8. Results from within-site genetic structure analysis (Chapter 3). Simulations were run for values of K ranging from 1-3. The most likely value of K, as interpreted following the guidelines of Pritchard et al 2001, is indicated here with an asterix. The proportion of individuals assigned to each cluster in each simulation are shown, as well as the allele frequency divergence between each of the clusters. ** Allele frequency divergence is based on Kullback-Liebler D and provides a measure of genetic distance between populations (Pritchard, Stephens and Donnelly 2000). A large value of D indicates greater genetic distance than a small one.

	K	Ln(PrD|K)	Proportion of individuals in each of the K=[1-3] clusters			Allele frequency divergence (D)** between each of the clusters		
			1	2	3	Clusters 1-2	Clusters 1-3	Clusters 2-3
Site 1	*1*	-2991.06	0.00			-		
	*2**	-2854.18	0.32	0.68		0.43	-	
	3	-2849.36	0.30	0.31	0.39	0.64	0.45	0.14
Site 2	*1*	-9402.8	0.00			-		
	*2**	-9067.4	0.39	0.61		0.67	-	
	3	-9242.1	0.35	0.34	0.31	0.00	0.70	0.69
Site 3	*1*	-11730.1	0.00			-		
	*2**	-11348.2	0.56	0.45		0.71	-	
	3	-11473.3	0.32	0.34	0.34	0.77	0.75	0.02
Site 4	*1*	-16471.7	0.00			-		
	*2**	-15600.7	0.54	0.46		0.61	-	
	3	-15663.0	0.39	0.31	0.30	0.88	0.83	0.03
Site 5	*1**	-2006.4	0.00			-		
	2	-2010.9	0.50	0.50		0.22	-	
	3	-2281.2	0.33	0.33	0.34	0.02	0.00	0.01
Site 6	*1**	-2019.5	0.00			-		
	2	-2051.2	0.50	0.50		0.15	-	
	3	-2276.2	0.34	0.33	0.33	0.01	0.04	0.01
Site 7	*1**	-10224.8	0.00			-		
	2	-10443.3	0.51	0.49		0.11	-	
	3	-10620.3	0.32	0.34	0.34	0.13	0.08	0.14
Site 8	*1**	-909.8	0.00			-		
	2	-914.1	0.49	0.51		0.15	-	
	3	-995.1	0.33	0.33	0.34	0.00	0.00	0.00